MONSANTO

Petition for the Determination of Nonregulated Status for Roundup Ready® Flex Cotton MON 88913

The undersigned submits this petition under 7 CFR Part 340.6 to request that the Administrator, make a determination that the article should not be regulated under 7 CFR Part 340.

Submitted by:

[redacted]

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25 March 2004

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OECD Unique Identifier: MON-88913-8

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No CBI

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Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner which are unfavorable to the petition.

[redacted] 3/25/2004

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Summary

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 CFR U.S.C. 7701-7772), to prevent the introduction and/or dissemination of plant pests into the United States or interstate introduction and/or dissemination. The APHIS regulations, (7 CFR § 340.6) provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Since the commercialization of the first cotton varieties with biotechnology traits in the mid-1990s, one of the most successful in terms of farmer adoption has been Roundup Ready® cotton event 1445 (hereinafter referred to as Roundup Ready cotton), which is tolerant to glyphosate, the active ingredient in Roundup® agricultural herbicides. Roundup Ready cotton, the first-generation herbicide-tolerant cotton product from Monsanto, was commercialized in the U.S. in 1997. Roundup Ready cotton has been widely adopted by cotton farmers and has made up a significant portion of the U.S. cotton production. Cotton varieties containing the Roundup Ready trait are currently cultivated on more than 7.8 million acres annually within the U.S. (USDA-NASS, 2003a).

Monsanto Company has now developed a second-generation glyphosate-tolerant cotton product, Roundup Ready Flex cotton MON 88913, that provides increased tolerance to glyphosate during the critical reproductive phases of growth compared to Roundup Ready cotton. Use of MON 88913 will enable the application of a Roundup agricultural herbicide over the top of the cotton crop at later stages of development than is possible with the current product. This will allow for effective weed control during crop production, because Roundup agricultural herbicides are highly effective against the majority of annual and perennial weeds that can be problematic during the later stages of crop development, with minimal risk of crop injury. The increased level of glyphosate tolerance in MON 88913 is achieved through the use of improved promoter sequences that regulate the expression of the *cp4 epsps* coding sequence.

MON 88913 was developed using the same *cp4 epsps* coding sequence and chloroplast targeting sequence and produces the same CP4 EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase) as Roundup Ready cotton. The transformation methodology used to produce MON 88913, *Agrobacterium tumefaciens*-mediated plant transformation, is

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comparable to the method used in development of Roundup Ready cotton. MON 88913 utilizes the same *cp4 epsps* coding sequence in the same crop, and confers the same glyphosate-tolerant phenotype as the nonregulated commercial Roundup Ready cotton product. The data presented in this petition characterize the nature and stability of the genetic modification, characterize the CP4 EPSPS protein produced in MON 88913 and demonstrate that MON 88913 is not phenotypically different than the negative segregant control, MON 88913(-).

As determined by Southern blot analysis, MON 88913 contains a single, intact DNA insert from the binary plasmid PV-GHGT35 at a single integration locus within the cotton genome. The DNA insert in MON 88913 contains two intact *cp4 epsps* gene expression cassettes containing identical *cp4 epsps* coding sequences. Polymerase chain reaction was performed to confirm the 5' and 3' insert-to-genomic DNA junctions and the organization of the elements within the insert in MON 88913. The DNA insert and the Roundup Ready trait are stable across multiple sexual generations. Phenotypic segregation data confirmed that the single insert locus and Roundup Ready trait behave as a single dominant locus with the expected Mendelian segregation pattern across multiple generations.

The production of CP4 EPSPS protein was confirmed by determining the level of CP4 EPSPS in tissues produced under field conditions. The levels of CP4 EPSPS protein were determined by validated enzyme-linked immunosorbent assay methods in leaf tissue sampled throughout the growing season, as well as in root, pollen and seed. The cotton plants were tolerant to over-the -top applications of a Roundup agricultural herbicide at later stages of development than is possible with cotton varieties that contain the Roundup Ready 1445 event.

The CP4 EPSPS protein produced in MON 88913 is targeted to the chloroplasts via an N-terminal fusion with the chloroplast transit peptide, CTP2, to form a CTP2-CP4 EPSPS precursor protein. The precursor protein produced in the cytoplasm is processed to remove the transit peptide upon translocation into the plant chloroplast, resulting in the mature CP4 EPSPS protein. The identity of the plant-produced protein was confirmed using data from western blot analysis and N-terminal sequence analysis. On the basis of western blot analysis, the electrophoretic mobility and immunoreactive properties of the plant-produced CP4 EPSPS protein were found to be equivalent to those of the *E. coli*-produced CP4 EPSPS reference standard protein.

Safety assessment of the CP4 EPSPS protein included protein characterization demonstrating the lack of similarity to known allergens and toxins, the long history of safe consumption of similar EPSPS proteins from a variety of food sources, CP4 EPSPS digestibility *in vitro*, and the lack of acute oral toxicity in mice. These data will be presented to the U.S. FDA for evaluation of food and feed safety for MON 88913 as part of the pre-market consultation process for products of modern biotechnology.

The phenotypic evaluation of MON 88913 included the key agronomic characteristics of seed germination, plant growth and development under field conditions, crop productivity

and harvest quality. These agronomic characteristics were evaluated by replicated field observations at 14 locations in 11 states across the U.S. cottonbelt to evaluate whether the presence of the DNA insert or the presence of the CP4 EPSPS protein altered the phenotypic characteristics and/or ecological interactions of MON 88913. In these evaluations, MON 88913 was compared to MON 88913(-), a negative segregant of MON 88913 that contains similar background genetics but does not contain the DNA insert. No differences were detected for 439 of a total of 458 comparisons between MON 88913 and MON 88913(-) by field location at $p \leq 0.05$. The majority of the 19 differences that were observed occurred for a single characteristic at a single field location. When all data were pooled across locations, only a single statistically significant difference in the growth and development characteristics was observed: the date until 50% flowering was slightly later for MON 88913 compared to MON 88913(-) (64 vs. 63 days after planting, respectively). This difference was one day at most sites and has no significant biological meaning in terms of plant pest potential. No differences between MON 88913 and MON 88913(-) were detected for any of the measured plant map characteristics: plant height, number of nodes, plant height per node, total number of bolls, number and quality of first and second position bolls, number of vegetative bolls, percent abnormal bolls, and the percent first and second position bolls at various nodal positions along the plant. A single difference was observed across sites in the boll/seed measurements. The seed index (grams per 100 seed) of MON 88913 was lower than MON 88913(-). This difference was approximately 0.3 g per 100 fuzzy seed, and likely has little biological meaning in terms of plant weed potential and the values fall well within the range for commercial cottonseed. No consistent trends for changes in seed number occurred when the data were pooled across locations. When boll and fiber quality data were analyzed, MON 88913 boll size was smaller and micronaire was less compared to MON 88913(-) (4.56 vs. 4.70 g per boll and 3.758 vs. 3.881 mike units, respectively). Small changes in seed size and micronaire are unlikely to increase weed potential, and both micronaire values are agronomically equivalent, falling within the premium target range of 3.7 – 4.2 for commercial cotton varieties.

Data were also collected on the presence of, and plant response to, in-field plant stressors such as pests and diseases. The ecological interactions data led to the conclusion that MON 88913 does not confer any detectable increase in the pest potential of cotton, nor were there any detectable unanticipated changes in the interactions between MON 88913 and the environment. These phenotypic data support the overall conclusion that there are no biologically meaningful changes present in MON 88913 beyond the intended Roundup Ready trait.

The nutritional composition of MON 88913 was compared to MON 88913(-) and sixteen commercial conventional cotton varieties using cottonseed collected from replicated field trials. The results of these compositional analyses show that, for the 53 components statistically evaluated, there were no statistically significant differences ($p \leq 0.05$) in 236 of the 265 comparisons made between MON 88913 and MON 88913(-). Of the 29 statistically significant differences, all MON 88913 values fell within the population of commercial conventional cottonseed as described by the 99% tolerance interval and/or within published ranges for conventional cottonseed. These data support the conclusion

that cottonseed of MON 88913 is compositionally equivalent to that of the cotton varieties grown commercially today.

The potential for outcrossing to sexually compatible related species is unlikely because populations of these related species in the U.S. are small and isolated from commercial cotton production areas. The environmental consequences of pollen transfer from MON 88913 to other cotton or other related *Gossypium* species is considered to be negligible. This is because of limited movement of cotton pollen, the safety of the introduced protein, and the lack of any selective advantage that would be conferred to recipient feral cotton or wild relatives if pollen transfer were to occur; these conclusions are consistent with the conclusions reached by USDA-APHIS in the Environmental Assessment and Finding of No Significant Impact for Roundup Ready cotton.

The agronomic consequences of volunteer cotton plants would be minimal as these plants are easily controlled by mechanical means or by one of a number of herbicides currently registered for control of cotton. Based on the data and information presented in this submission, the previous USDA conclusions for Roundup Ready cotton, and the extensive experience gained during the marketing of Roundup Ready cotton, it is concluded that the anticipated environmental consequences of the introduction of MON 88913 would be negligible, and there is no reason to believe that MON 88913 would have an adverse impact on organisms beneficial to plants or to "nontarget" organisms, including threatened or endangered organisms. The introduction of MON 88913 is expected to enhance the economic, environmental, and superior weed control benefits afforded by the current product, Roundup Ready cotton.

Data and information presented in this request demonstrate that MON 88913 does not represent a unique plant pest risk. Therefore, Monsanto Company requests a determination from APHIS that Roundup Ready Flex cotton MON 88913, and all progeny derived therefrom, be no longer considered regulated articles under regulations in 7 CFR § 340.

Abbreviations and Definitions

~	Approximately
A	Acre
AA	Amino acid
aad	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase, from the transposon Tn7
ADF	Acid detergent fiber
Ae	Acid equivalent
ANOVA	Analysis of Variance
AOSA	Association of Official Seed Analysts
APS	Analytical protein standards
Avg	Average
B	Border region
BSA	Bovine serum albumin
C	Celsius
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CI	Confidence interval
CP4	*Agrobacterium sp.* strain CP4
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* species strain CP4
cp4 epsps	Coding sequence for the CP4 EPSPS protein from *Agrobacterium sp.* strain CP4 present in PV-GHGT35
CR	Coding region
CTP	Chloroplast transit peptide
ctp2	Chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
DAP	Days after planting
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
DWCF	Dry weight conversion factor
dwt	Dry weight
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
FA	Fatty acid
FDA	United States Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	Figwort mosaic virus
fwt	Fresh weight
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
I	Intron
I-ACT8	Intron and flanking exon sequence from the *act8* gene of *Arabidopsis thaliana*
IgG	Immunoglobulin G
I-TSF1	Intron from the *Arabidopsis thaliana tsf1* gene encoding elongation factor EF-1alpha
IUPAC-IUB	International Union of Pure and Applied Chemistry - International Union of Biochemistry
Kb	Kilobase pair
KCl	Potassium chloride
K_I	Inhibition constant
K_M	Michaelis constant
L	Leader
L-ACT8	Leader sequence from the *act8* gene of *Arabidopsis thaliana*
LB	Left border
LOQ	Limit of quantitation
LOD	Limit of detection
L-TSF1	Leader (exon 1) from the *Arabidopsis thaliana tsf1* gene encoding elongation factor EF-1alpha
$MgCl_2$	Magnesium chloride
Mike	Micronaire
MOA	Mode of action
MW	Molecular weight
na	Not available
NaCl	Sodium chloride
NaOAc	Sodium acetate
NDF	Neutral detergent fiber
NFDM	Non-fat dried milk
NIST	National Institute of Standards and Technology
OD	Optical density
OSL	Overseason leaf
OR	Origin of replication
OR-ORI-PBR322	Origin of replication from pBR322 for maintenance of plasmid in *E. coli*
OR-ORI V	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2

Overseason leaf	Leaf material collected from different time points during the growing season
P	Promoter
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% (v/v) Tween-20
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
P-FMV/TSF1	Chimeric promoter containing the *Arabidopsis thaliana tsf1* gene promoter, encoding elongation factor EF-1alpha, and enhancer sequences from the Figwort Mosaic virus 35S promoter
ppm	Parts per million (µg of analyte/g of sample)
P-35S/ACT8	Chimeric promoter containing the promoter of the *act8* gene of *Arabidopsis thaliana* combined with the enhancer sequences of the Cauliflower mosaic virus (CaMV) 35S promoter
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
qt	Quart
RB	Right border
rbc	Ribulose-1, 5-bisphosphate carboxylase
Rop	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
RQTY	Relative quantity
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
sp	Species
S3P	Shikimate-3-phosphate
TBA	Tris-borate buffer with L-ascorbic acid
TDF	Total dietary fiber
T-DNA	Transfer(ed) DNA
TE	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
T-E9	DNA sequences derived from *Pisum sativum,* containing the 3' nontranslated region of the pea ribulose-1, 5-bisphosphate carboxylase, small subunit E9 gene
TI	Tolerance interval
Tris	Tris(hydroxymethyl)aminomethane
TS	Targeting sequence
TSSP	Tissue-specific site pool
USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service

Standard abbreviations, e.g., units of measure, are be used according to the format described in 'Instructions to Authors' in the *Journal of Biological Chemistry*

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I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR Part 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167), to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR § 430.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

I.B. Rationale For the Development of Roundup Ready Flex Cotton, MON 88913

This section provides background and rationale for the development of Roundup Ready Flex cotton, MON 88913, including benefits of the technology, anticipated herbicide use and farmer adoption. MON 88913 is a second-generation glyphosate-tolerant cotton product, which provides increased tolerance to glyphosate relative to the current product, Roundup Ready cotton event 1445, (hereinafter referred to as Roundup Ready cotton), to provide more effective and flexible weed control options during production.

Control of weeds in a cotton crop is essential because weeds compete with the crop for the same limited resources in the field including sunlight, water and nutrients (Ross and Lembi, 1985; Wilcut et al., 2003). Because failure to control weeds within the crop can result in decreased yields and reduced crop quality, an intensive program for weed control is essential to ensure profitability (Wilcut et al., 2003; Hayes et al., 2001). Losses from weeds result in a $300 million crop loss per year (Abernathy and McWhorter, 1992). In addition, weeds present at cotton harvest reduce the efficiency of the mechanical harvest of the crop and can reduce both the quality and value of the lint because of staining by vegetation.

Current weed management systems interweave cultural and mechanical practices with herbicides to overcome the competitive effect of the weeds. In general, weeds must be controlled both before emergence and shortly after crop emergence to avoid yield loss. Early control is especially important because weeds that emerge early relative to the cotton crop are more competitive and thus cause greater crop loss than weeds that emerge later in the season (Coble and Byrd, 1993). Because of this, cotton should be kept as weed-free as possible from emergence through at least eight weeks.

Use of a Roundup agricultural herbicide in crop provides an efficient and cost-effective means of controlling weeds. Roundup agricultural herbicides are used as foliar-applied, nonselective herbicides, and are effective against the majority of annual and perennial grasses and broad-leaf weeds. Glyphosate, the active ingredient in Roundup agricultural

herbicides, binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of 5-enolpyruvylshikimate-3-phosphate, thereby starving plants of essential amino acids and secondary metabolites (Steinrucken and Amrhein, 1980; Haslam, 1993). EPSPS proteins catalyze the transfer of the enolpyruvol group from phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (Alibhai and Stallings, 2001). In Roundup Ready plants, which are tolerant to Roundup agricultural herbicides, aromatic amino acids and other metabolites that are necessary for plant growth and development are met by the continued action of the inserted glyphosate-tolerant CP4 EPSPS enzyme (Padgette et al., 1996).

Glyphosate has favorable environmental and safety characteristics. Glyphosate has no preemergence or residual soil activity (Franz et al., 1997) and is not prone to leaching, degrades in soil over time, and poses no unreasonable risks to mammals, birds or fish under normal use conditions (U.S. EPA, 1993; WHO, 1994; Giesy et al., 2000; Williams et al., 2000). Furthermore, glyphosate has been extensively evaluated in scientific studies that have concluded that glyphosate does not cause cancer, birth defects, mutagenic effects, nervous system effects or reproductive problems (Williams et al., 2000).

The first biotechnology cotton products were commercially launched in 1995 (BXN® cotton) and 1996 (Bollgard® cotton). On July 11, 1995, USDA-APHIS determined the nonregulated status (Fed. Reg. 60:37870-37871) of the first-generation glyphosate-tolerant cotton product, Roundup Ready cotton, which was subsequently commercialized in 1997. Since then, Roundup Ready cotton has been rapidly adopted by U.S. cotton farmers (95% grower satisfaction, Monsanto unpublished survey results) and has been a significant part of U.S. annual cotton production since its market introduction. Cotton with the Roundup Ready trait is currently cultivated on approximately 59% of the U.S. cotton acres (USDA-NASS, 2003b). However, a constraint within the current Roundup Ready cotton system is the limitation of in-crop, over-the-top herbicide application to Roundup Ready cotton plants with no more than four true leaves. Applications at the fifth true leaf stage and beyond require specialized spray equipment to aim the herbicide between the rows and away from the cotton plant.

Subsequently, Monsanto Company has developed a second-generation glyphosate-tolerant cotton product, Roundup Ready Flex cotton, MON 88913, that provides increased tolerance to glyphosate compared to the current product, Roundup Ready cotton. Use of MON 88913 will enable the application of a Roundup agricultural herbicide over the top of the cotton crop at later stages of development than is possible with Roundup Ready cotton. This will provide more effective weed control during crop production with minimal risk of crop injury because Roundup agricultural herbicides are highly effective against the majority of annual and perennial weeds that can be problematic during the later stages of crop development. The increased level of glyphosate tolerance in MON 88913 is achieved through use of improved promoter

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sequences that regulate the expression of the *cp4 epsps* coding sequence (Fincher et al., 2003).

MON 88913 was developed using the same *cp4 epsps* coding and chloroplast targeting sequences as the current Roundup Ready cotton product and produces the same CP4 EPSPS protein that confers the Roundup Ready trait. The same transformation methodology used to produce MON 88913 was also used in development of Roundup Ready cotton. Thus, MON 88913 contains the same *cp4 epsps* coding sequence in the same crop, and confers the glyphosate-tolerant phenotype similar to the commercial Roundup Ready cotton, but with increased tolerance to glyphosate during the sensitive reproductive stages of growth.

MON 88913 will be introduced through conventional breeding new cotton varieties in anticipation of commercialization. Field experiments with MON 88913 were conducted in 2000 - 2003 throughout the U.S. cotton growing regions under USDA notification (Appendix A). Results from these field trials have demonstrated that MON 88913 is tolerant to over-the-top applications of a Roundup agricultural herbicide through the layby stage (canopy closure into the row) and beyond, with the expected level of weed control.

Product characterization studies summarized in this submission demonstrate that, with the exception of increased production of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (CP4 EPSPS), MON 88913 is not different than cotton currently grown in the U.S. There is no evidence that growing MON 88913 cotton will result in any adverse effects to the environment. In addition, agronomic evaluations of plant vigor, growth habit characteristics, and general disease susceptibility have shown MON 88913 not to be different than negative segregant control plants that were derived from MON 88913 but do not contain the DNA insert. Use of a Roundup agricultural herbicide over the top of MON 88913 would not be expected to cause any adverse changes in the field environment outside of the current cotton production system using Roundup Ready cotton varieties. The commercialization of MON 88913, following receipt of required regulatory clearances, including a determination of nonregulated status from USDA-APHIS, will represent an efficacious and environmentally compatible addition to the existing options for weed control in cotton.

The introduction of MON 88913 is expected to continue to provide the grower with economic and environmental benefits and superior weed control benefits to those currently provided by Roundup Ready cotton. These benefits include:

1. Effective weed control: The Roundup Ready cotton system provides farmers with effective weed control and equivalent yields while reducing the number of herbicide applications required (Culpepper and York, 1998, 2000; Gianessi et al., 2002a). Growers experience improved flexibility in weed control compared to herbicide programs used in conventional cotton, as specific preemergent herbicides that are used for prevention are replaced by a broad-spectrum post-emergent herbicide that can be used on an as needed basis (Welch et al., 1997; Culpepper and York, 1998).

2. Convenience and simplicity: The Roundup Ready cotton system increases farming convenience and production simplicity (Culpepper and York, 1998; McCloskey, 1998), which was a major driver for the adoption of Roundup Ready cotton (Kalaitzandonakes and Suntornpithug, 2001). Additionally, the Roundup Ready cotton system offers crop rotation options over other weed control systems, is an easier system to manage, and more acreage can be covered by the same equipment (Kalaitzandonakes and Suntornpithug, 2001; Culpepper and York, 1998; McCloskey, 1998). Less labor is often required because of the elimination of hand weeding and the high cost of early, postdirected sprays that require special equipment (McCloskey et al., 1998).

3. Increased grower income: Use of Roundup Ready cotton has shown reduced production costs, net economic advantage, and reduced production risks (Gianessi et al., 2002a; Kalaitzandonakes and Suntornpithug, 2001). In 2001, herbicide-tolerant cotton increased the total net value of U.S. cotton production by $133 million dollars (Gianessi et al., 2002a).

4. Increased adoption of reduced tillage practices: Use of the Roundup Ready cotton system encourages adoption of reduced tillage practices by growers (Gianessi et al., 2002; Kalaitzandonakes and Suntornpithug, 2001). It is estimated that reduced tillage is practiced on one out of every two new acres of Roundup Ready cotton (Kalaitzandonakes and Suntornpithug, 2001). The use of conservation tillage practices reduces water runoff by 30% compared to conventional tillage practices, thereby improving the quality of surface water (Baker and Johnson, 1979). Additionally, conservation tillage improves water quality and creates habitat for wildlife (CTIC, 1999; Fawcett and Towry, 2002). Use of the Roundup Ready cotton system significantly improves overall weed control in conservation tillage cotton (Keeling et al., 1998).

5. Compatibility with Integrated Pest Management (IPM) and soil conservation techniques: Roundup Ready cotton is highly compatible with integrated pest management and soil conservation techniques (Keeling et al, 1998; Patterson et al, 1998; Smart and Bradford, 1999), resulting in a number of important environmental benefits including reduced soil erosion and improved water quality (Baker and Laflen, 1979; Hebblethewaite, 1995; CTIC, 1998), improved soil structure with higher organic matter (Kay, 1995; CTIC, 2000), improved wildlife habitat (Phatak et al., 1999), improved carbon sequestration (Reicosky, 1995; Reicosky and Lindstrom, 1995), and reduced CO_2 emissions (Kern and Johnson, 1993; CTIC, 2000).

6. History of safe use: The U.S. EPA (1993) has concluded that the use of Roundup agricultural herbicides does not pose unreasonable risks to humans, birds, mammals, aquatic organisms, bees and invertebrates. Glyphosate, the active ingredient in Roundup agricultural herbicides, has favorable environmental characteristics compared to some other herbicides (Nelson and Bullock, 2003).

In addition to the current benefits of the Roundup Ready cotton system, the second-generation cotton product, MON 88913, is expected to provide additional grower benefits. MON 88913 will offer growers an expanded window for application of Roundup agricultural herbicides and enhanced flexibility in weed control options relative to the current Roundup Ready cotton product. Roundup Ready cotton currently allows over-the-top applications of a Roundup agricultural herbicide through the fourth leaf (node) stage, and thereafter is limited to post-directed sprays up to layby. Further, at least ten days and two nodes of incremental growth also must occur between applications because of reproductive tolerance limitations. Although excellent weed control is obtained with the Roundup Ready cotton system within these application constraints, MON 88913 will provide growers with an improved version of Roundup Ready cotton with enhanced reproductive tolerance for greater flexibility in weed control options with minimal risk of crop injury.

The key anticipated added benefits of MON 88913 relative to the current Roundup Ready cotton product include:

1. Enhanced grower convenience with season-long application options. Further, the expanded over-the-top window allowed by the use of this technology is expected to increase production efficiency. Growers will have the option to schedule Roundup agricultural herbicide applications with insecticide applications and plant growth regulators common to cotton production, reducing labor and equipment costs.

2. Potential for simplification of spray equipment. For example, reduced need to purchase specialized spray equipment necessary to apply a Roundup agricultural herbicide after the four-leaf stage.

3. Potential for greater weed control efficacy by a reduction of weed control application complications brought on by weather and equipment failure. Over-the-top applications in crop have the potential to provide improved weed control compared to precision post-directed applications.

4. Enhanced margin of assured crop safety with Roundup-based herbicide applications.

5. Fewer challenges integrating weed control measures with irrigation activities.

6. Enhanced ability to tailor herbicide applications to weed development stage instead of to the cotton developmental stage.

Effective weed control and crop management is critical to maximizing cotton yield and retaining a high-quality harvest, free of weedy material. For effective weed control, growers typically select a herbicide based on several factors including the weed species present, weed size, weed population, risk of potential crop injury, and the cost of the herbicide program. Also important are application convenience, compatibility with other

crop chemicals, and environmental characteristics. Although few herbicides deliver optimal performance in all of these areas, use of a Roundup agricultural herbicide in a Roundup Ready cotton system provides the grower with broad-spectrum weed control, including the majority of economically important annual and perennial grasses and broad-leaf weeds (Wilcut et al., 2003). In addition to Roundup agricultural herbicides, a wide range of weed control options are available and utilized, including other herbicide products are currently employed in cotton production to achieve sustainable weed management (manuals published by state extension offices: University of Tennessee, 2004; Baumann, 1998; Mississippi State University, 2004).

Weed control systems used in U.S. cotton production are reviewed in detail in Section VIII. Crop rotational practices in cotton and use of multiple herbicide modes of action are also presented in more detail in Section VIII. Weed control in cotton is typically a two-to-four application process over the length of the growing season. The choice of weed-control methods depends on the particular cropping system (e.g., conventional-tillage, conservation-tillage, no-till), local weed spectrum, costs, and other factors already mentioned. A Roundup agricultural herbicide may be tank-mixed with herbicides that provide residual activity, or alternatively, followed by other in-crop herbicides using hooded post-directed sprayers to achieve optimum weed control on tough-to-control species (for examples see Wilcut et al., 2003). MON 88913 is expected to utilize similar weed control programs compared to those already used in Roundup Ready cotton, including a combination of herbicide sprays with varied chemical modes of action. However, the timing of Roundup agricultural herbicide applications would not be as restrictive as those labeled for the current product, Roundup Ready cotton (see Section VIII.).

In conclusion, weeds are a severe constraint that must be managed in the production of cotton as cotton cannot compete effectively in its early growth stages and must be protected from the invasion of aggressive weeds. Current management systems combine cultural and mechanical practices with herbicides to overcome the competitive effect. The introduction of the current Roundup Ready cotton product has reduced the number and quantity of herbicide applications, resulting in more flexible options for effective control of the weeds. The use of a Roundup Ready cotton system also offers environmental benefits associated with the use of conservation tillage and integrated weed-management practices. The second-generation glyphosate-tolerant cotton product, Roundup Ready Flex cotton, MON 88913, provides increased tolerance to glyphosate compared to the current commercial product. Use of MON 88913 will enable the application of a Roundup agricultural herbicide over the top of the cotton crop at later stages of development than is currently possible with Roundup Ready cotton. This will provide more effective weed control during crop production with minimal risk of crop injury. Roundup Ready Flex cotton, MON 88913, will provide growers with a new, more flexible Roundup Ready cotton system that provides excellent weed control using familiar weed control management practices that are fully compatible with conservation tillage practices, while retaining all the current grower and environmental benefits of the Roundup Ready cotton system.

I.C. Adoption of MON 88913

Currently, cotton growers in the U.S. have a wide range of weed control options when they make planting decisions, both biotechnology and conventional-based. Assuming significant demand for greater weed control convenience, a wider application window, enhanced weed control flexibility, and other benefits in using the second-generation Roundup Ready Flex technology, Monsanto anticipates that MON 88913 will rapidly replace a majority of the acres on which Roundup Ready cotton is currently grown.

I.D. Submissions to Other Regulatory Agencies

Submission to FDA
MON 88913 is within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA, 1992). In compliance with this policy, Monsanto will submit to FDA a food and feed safety and nutritional assessment summary for Roundup Ready Flex cotton, MON 88913.

Submission to EPA
The United States Environmental Protection Agency has authority over the use of pesticidal substances, under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), as amended (7 U.S.C. § 136 *et seq.*). A submission of glyphosate residue data and proposed labeling for the expanded use of Roundup UltraMAX® herbicide (EPA Reg. No. 524-512) on Roundup Ready Flex cotton, MON 88913, was made to the EPA in March, 27, 2003.

Pursuant to section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), 21 U.S.C. 346 a(d), the EPA has previously reviewed and established an exemption from the requirement for a tolerance for the CP4 EPSPS protein and the genetic material necessary for the production of this protein in or on all raw agricultural commodities (40 CFR § 180.1174).

Submissions to Foreign Governments
Regulatory submissions for import and production approvals will be made to countries that import U.S. cottonseed and have regulatory approval processes in place. These will include submissions to the Japanese Ministry of Health, Labor and Welfare and Ministry of Agriculture, Forestry and Fisheries, Health Canada and the Canadian Food Inspection Agency.

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II. The Cotton Family

This section describes the biology of the cotton crop. In addition to the material provided below, all general aspects of the biology, genetics, and agronomy of the cotton crop relevant to this petition were previously submitted to the Agency by Monsanto as part of the Bollgard cotton, Bollgard II® cotton and Roundup Ready cotton petitions (94-308-01P, 00-342-01P and 95-045-01P, respectively).

II.A. Cotton as a Crop

Four species of the genus *Gossypium* are known as cotton, which is grown primarily for the fiber produced from the seed coat trichomes that is made into textiles. Cotton is the leading plant fiber crop produced in the world and the most important in the U.S. Commercial cotton has been extensively characterized and has a long history of agricultural production (Supak et al., 1992; USDA, 2001; USDA-ERS, 2003a; USDA 2003a). A short review of the biology and use of cotton in the United States is available from the USDA-APHIS at http://www.aphis.usda.gov/brs/.

In the U.S., commercial cotton has a long history of agricultural production (USDA, 2001; USDA-ERS, 2003a; USDA 2003a). Cotton production in the U.S. is located primarily in a region including 17 southern states across the cottonbelt, which extends across the southern and western U.S. from Virginia south and west to California. Cultivated cotton is noted for its general adaptability and high productivity and *G. hirsutum* is the predominant species grown in the U.S. and globally (Lee, 1984). Cotton fiber is used for cordage and other nonwoven products, as well as for textiles. In addition, cotton linters, which are the short fibers removed from seeds prior to crushing, are a major source of industrial cellulose.

In addition to cotton lint, cottonseed meal and oil are produced as valuable byproducts. Cottonseed is also used in manufacturing cottonseed oil as a premium quality oil used for a variety of food uses, including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil. Cottonseed meal and hulls from the seed are not used for human consumption, but principally are sold as feed for livestock. The short fibers on the cottonseed, or linters, consist primarily of cellulose. The linters, after extensive processing, are used in a wide variety of food and industrial products (NCPA, 1990). Linter fiber is used to improve the viscosity of food dressings. Viscose, as a food-use product, is utilized in bologna and sausage casings, but is also used in the manufacture of rayon.

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II.B. The Taxonomy of Cotton

Cotton belongs to the genus *Gossypium* of the tribe Gossypieae of the family Malvaceae of the order Malvales (Fryxell, 1979; Munro, 1987). Some authors have grouped species differently, and *Gossypium* has been included in the tribe Hibisceae (Smith, 1977). The genus *Gossypium* is currently comprised of 49 species that are widely distributed and occur predominately in tropical and subtropical regions around the world (Percival et al., 1999). The taxonomic status of a number of noncultivated species, especially in Africa and the Middle East, is still under development. Several primary centers of diversity have developed, and the greatest species diversity occurs in northwestern Australia, North Eastern Africa, and the Arabian Peninsula, and the western and northern part of Mexico (Percival et al., 1999).

Worldwide, four *Gossypium* species are collectively known as cotton and are grown commercially. These include two diploid species (2n=2x=26) *G. arboreum* L. and *G. herbaceum L.,* which evolved in Africa and the Middle East, and two allotetraploid species (2n=4X=52) *G. barbadense* and *G. hirsutum,* which evolved in the Americas (reviewed in Brubaker et al., 1999; Percival et al., 1999; Supak et al., 1992).

There are four species of *Gossypium* in the U.S. Two of them, *G. hirsutum* (upland cotton) and *G. barbadense* (pima), are introduced species and are grown commercially. In addition, wild populations of *G. hirsutum* are known to occur in southern Florida ([redacted] personal communication). The two species native to the U.S. are *G. thurberi* Todaro and *G. tomentosum* Nuttall ex Seeman (Brown and Ware, 1958; Fryxell, 1979; Munro, 1987).

Gossypium thurberi Todaro (*Thurberia thespesiodes* Gray) is found in the mountainous regions of southern Arizona in the counties of Graham, Gila, Pinal, Maricopa, Cochise, Santa Cruz and Pima, and also in the Bradshaw Mountains of Yavapai County (Fryxell, 1979). *G. thurberi* is generally found at elevations of 2,500 to 5,000 feet and is isolated from areas of cotton production. Any gene exchange between this species and tetraploid cotton, if it were to occur, would result in triploid (3x=39) sterile plants. *G. hirsutum* and *G. barbadense* are allotetraploids (4x=52) and *G. thurberi* is a diploid (2x=26). Such sterile hybrids have been produced under controlled laboratory conditions, but would not be able to propagate in the wild. In addition, fertile allohexaploids (6x=78) have not been reported in the wild ([redacted] personal communication).

G. tomentosum is a tetraploid and is found on Hawaii (Degener, 1946). The local range is on the larger islands as well as on Nihau and Kahoolawe. It grows on arid, rocky, or clay plains not far from the sea. Thus, on the larger islands, it is found chiefly on the dry, leeward side. On Oahu it is common near Koko Crater, and grows scattered between Honolulu and Markus Balley. On Molokai it is common on the southwestern end; elsewhere it is rare except near Kamalo. On Maui the species may be found from the sea in one of the valleys south of Wailuku.

Worldwide, cotton taxonomy still remains to be fully elucidated; however, the phylogeny of the two commercial species in the U.S. is well established. Because of the purposeful

selection and transport of *Gossypium* species by humans over thousands of years in order to develop a high-quality and high-yielding marketable plant, "its morphology, genetic composition, and indigenous ranges have been altered significantly by human activity," basically transforming perennial shrubs or trees into a compact annual row crop producing a high-quality white fiber (Brubaker et al., 1999).
Improved modern varieties of *G. hirsutum* and *G. barbadense* are currently cultivated in overlapping regions of the southern U.S., with *G. barbadense* grown primarily in the western states of Arizona, California, New Mexico, and Texas, and *G. hirsutum* produced throughout the 17 states comprising the U.S. cottonbelt. *G. hirsutum* comprises the vast majority of U.S. cotton production, 13.7 million acres, compared to *G. barbadense* varieties, which were cultivated on less than 250,000 acres in 2002. The Code of Federal Regulations references cottonseed as an agricultural commodity without species distinctions. Additionally, in 7 CFR § 361.1 under definitions for seed used for seeding purposes in the U.S., cottonseed is defined as *Gossypium* sp. as a group, and not as individual cotton species.

II.C. The Genetics of Cotton

Phylogenetic classifications of the *Gossypium* genus have expanded in the last decade. There are three major lineages of the diploid *Gossypium* species: Australian (C, G, K genomes), the American continents (D genome), and Africa/Middle East (A, B, E, F genomes) (Percival et al., 1999).

The tetraploid species (2n=4x=52) including *G. hirsutum*, *G. barbadense* and *G. tomentosum* (in Hawaii) are comprised of the A and D nuclear genomes (AADD) and contain only the A chloroplast genome, indicating the seed parent of the original hybridization was of African or Middle Eastern descent (Percival et al., 1999). Diploid species, AA, BB, *etc.* (2n=2x=26), are distributed among tropical and subtropical regions worldwide. As mentioned above, two of the diploid species, *G. herbaceum* and *G. arboreum,* are of regional agronomic importance outside of the U.S.

Among cultivated cotton (*G. arboreum, G. herbaceum, G. hirsutum* and *G. barbadense*), introgression within ploidy/genome type is historically common because of expansion of the natural range through human intervention and cultivation. Interspecific exchange of genes is responsible for some of the genetic diversity found within each cultivated species (Brubaker et al., 1999).

II.D. Pollination of Cotton

Although natural crossing can occur, cotton is normally considered to be a self-pollinating crop (Niles and Feaster, 1984). The pollen is heavy and sticky and transfer by wind is unlikely. Regardless, there are no morphological barriers to cross-pollination based on flower structure. Pollen is transferred instead by insects, in particular by various wild bees, bumble bees (*Bombus* sp.), and honeybees (*Apis mellifera*). The range over which natural crossing occurs is limited. McGregor (1976) traced movement of

pollen by means of fluorescent particles and found that, even among flowers located only 150 to 200 feet from a cotton field that was surrounded by a large number of bee colonies to ensure ample opportunity for transfer of pollen, fluorescent particles were detected on only 1.6% of the flowers. For the sake of comparison, the isolation distances for foundation seed are 1320 feet, and for certified cottonseed and registered seed are 660 feet (7 CFR § 201).

Based on information previously submitted by Monsanto, the USDA stated in the environmental assessment documents for Bollgard cotton and Roundup Ready cotton that the "potential for gene introgression from genetically engineered cotton lines into wild or cultivated sexually compatible plants is very low" (USDA, 1995a, 1995b). Similarly, in the environmental assessment for Bollgard II cotton, the USDA stated "APHIS believes that it is very unlikely that cotton event 15985 will successfully cross with wild sexually compatible relatives when grown in the United States" (USDA, 2002). Recently, the USDA made the same determination on another herbicide tolerant cotton product, LLCotton 25 (USDA, 2003b). Importantly, the environmental consequences of pollen transfer from MON 88913 to other cotton or related *Gossypium* species is considered to be negligible because of the limited movement of cotton pollen and the lack of any selective advantage that would be conferred on the recipient feral cotton or wild relatives.

II.E. Weediness of Cotton

Cultivated cotton is ineffective as a weed. The USDA has previously determined that "cotton is not considered to be a serious, principal or common weed pest in the U.S." (USDA, 1995a). Cotton appears to be somewhat opportunistic towards disturbed land and appears not to be especially effective in invading established ecosystems. Cotton does not persist where freezing conditions occur and therefore there are only a few regions in the U.S. where cotton is capable of overwintering. Hence, in the continental U.S., wild populations of *G. hirsutum* exist only in the southern tip of Florida. The 2002 USDA Environmental Assessment for Bollgard II cotton states: "*Gossypium hirsutum* is not typically considered a weed species in the United States or other countries" (USDA, 2002), nor is *G. hirsutum* listed in the Southern Weed Science Society's Composite list of Weeds (1998). The Southern Weed Science Society lists *G. hirsutum* as a potential weed in southern Florida (Southern Weed Science Society, 1998); however, southern Florida is not a location where commercial cotton is cultivated. Feral populations of cultivated *G. hirsutum* and 'wild' populations of *G. hirsutum* race *'yucatanense'* are known to occur in South Florida and Puerto Rico ([redacted] personal communication) and would be capable of crossing with cultivated cotton, but they are not known to exist in cotton growing areas. The biogeography of cotton and outcrossing potential are discussed further in Sections VIII.B.3.A. and VIII.B.3.B. on the ecological assessment of MON 88913.

II.F. Characteristics of the Recipient Plant

The cotton variety used as the recipient for the DNA insert in MON 88913 was Coker 312. Coker 312 is an older commercial variety of upland cotton (*G. hirsutum*) and is the same recipient variety used for development of the current commercial Roundup Ready cotton.

II.G. Cotton as a Test System in this Petition

In developing the data in support of this petition, appropriate test and control materials were developed and, where feasible, use of commercial conventional reference cotton materials were used to establish a range of expected responses for commercial cotton in the U.S. Cotton, unlike hybrid crops, is a varietal crop in the U.S., and exhibits a significant amount of seed-to-seed genetic variability within a given commercial variety. This variability is a natural genetic resource effectively utilized by commercial cotton breeders. Thus, the production of positive inbreds (test) and negative inbreds or true isolines (control), commonly utilized for hybrid crops, are not necessarily feasible for cotton. In this regard, taking advantage of conventional genetics, negative segregants derived from the genotype-positive MON 88913 were developed as appropriate controls [MON 88913(-)] for field tests and related product characterization studies.

MON 88913(-) plants used for the characterization data in this petition were selected at the R2 stage where they were segregating for the DNA insert. MON 88913 was first identified at the R0 stage in the growth chamber and greenhouse by antibody strip tests that identify the presence of the CP4 EPSPS protein. These results were confirmed by PCR analysis specifically designed to detect the DNA insert in MON 88913.

MON 88913(-) plants were identified at the R2 stage by negative results in the antibody strip test and by PCR analysis specifically designed to detect the DNA insert in MON 88913. The genetic background of MON 88913(-) is expected to be very close, but not 100% identical, to that of MON 88913. Therefore, MON 88913(-) was considered a more appropriate negative control material than the generic conventional cottonseed of the recipient variety (Coker 312).

III. Description of the Transformation System

MON 88913 was produced using an *Agrobacterium*-mediated transformation system. This process has been generally described by Umbeck et al., (1987). *Agrobacterium tumefaciens* strain ABI (Monsanto proprietary strain), a derivative of *A. tumefaciens* strain C58 harboring PV-GHGT35 (Figures V-1a, b, Section V.), was the transformation vector. *A. tumefaciens* strain ABI contains a disarmed Ti plasmid that is incapable of inducing tumor formation due to a deletion of the phytohormone genes originally in the *Agrobacterium* Ti plasmid (Koncz and Schell, 1986). Briefly, this vector was cocultured with hypocotyl explants of *in vitro* cotton seedlings which were then used to generate somatic embryogenic cotton callus (generally following procedures described by Umbeck et al., (1987) (see Figure III-1). The callus was selected *in vitro* for the desired sectors by incorporating glyphosate into the culture medium. The *Agrobacterium* vector was eliminated from the cultures by incorporating antibiotics (carbenicillin and cefotaxime) into the culture medium. This process is comparable to the transformation method used to develop commercial Roundup Ready cotton, except that MON 88913 was selected *in vitro* using glyphosate as the selective agent, whereas Roundup Ready cotton was selected *in vitro* using kanamycin.

Glyphosate-tolerant callus produced somatic embryos that germinated and developed into plants. The resulting plants were further screened for commercial potential over several years in the growth chamber, greenhouse, and replicated field trials (Figure III-1). MON 88913 is derived from a single regenerated plant from the *Agrobacterium*-mediated transformation and regeneration process. MON 88913 was screened for glyphosate tolerance, field performance, crop familiarity, and aspects of commercial potential and entered into a pre-commercial backcrossing program (Figure III-2).

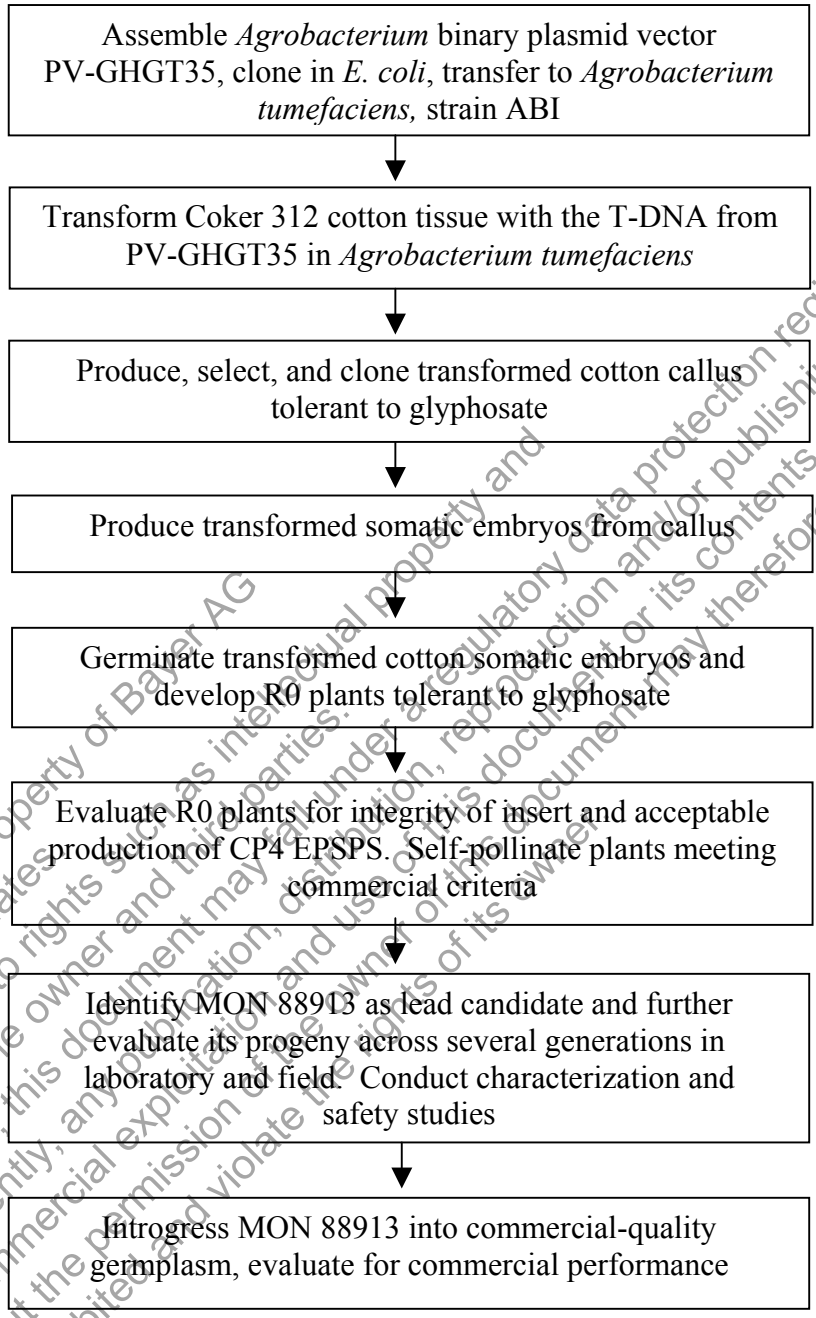


Figure III-1. Steps in the Development of MON 88913.

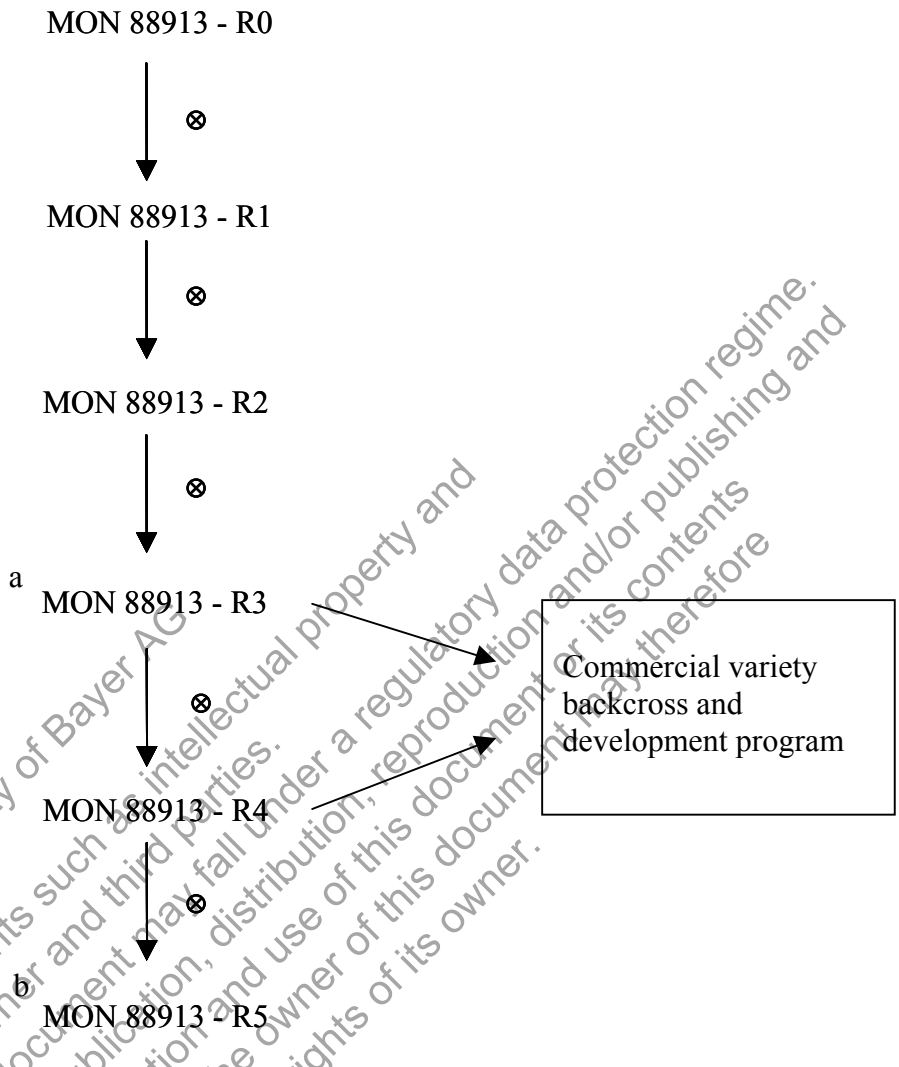


Figure III-2. Breeding Tree for Development of MON 88913.
Generations R1 through R5 were selected for generational stability by Southern blot analyses. Generations R3 and R4 were used as donors for commercial variety development.

R0 = Initial MON 88913 plant
⊗ = Crossed to self
a = Generation used for seed composition, molecular characterization, protein characterization, seed germination, and protein level determinations
b = Generation used for replicated agronomic field tests

IV. Donor Genes and Regulatory Sequences

This section describes the DNA materials used in the development of MON 88913 and the deduced amino acid sequence of the resulting CP4 EPSPS protein produced in MON 88913.

IV.A. Vector PV-GHGT35

MON 88913 was developed through *Agrobacterium*-mediated transformation of cotton hypocotyl tissue using the double-border, binary vector PV-GHGT35 shown in Figures V-1a, b. This vector contains two joined *cp4 epsps* gene expression cassettes delineated by left and right border regions. This T-DNA of approximately 8.2 kb contains two tandem *cp4 epsps* gene expression cassettes which were transferred into the cotton genome by *Agrobacterium tumefaciens* during the *in vitro* transformation process. From the right border region, the first *cp4 epsps* coding sequence is under the regulation of a chimeric transcriptional promoter P-FMV/TSF1, TSF1 leader and intron sequences, a chloroplast transit peptide (TS-*ctp2*) sequence and a T-E9 polyadenylation sequence. The second *cp4 epsps* coding sequence is regulated by a P-35S/ACT8 chimeric transcriptional promoter, L-Act8 leader and intron sequences, and the same chloroplast targeting and polyadenylation sequences as utilized in the first *cp4 epsps* gene expression cassette. The *cp4 epsps* coding sequence used to produce MON 88913 is the same as that employed in the current Roundup Ready cotton product, which has previously been granted deregulated status by the USDA. A description of the genetic elements in vector PV-GHGT35 is provided in Table IV-1.

IV.B. The *cp4 epsps* Coding Sequence and CP4 EPSPS Protein

The *cp4 epsps* gene from *Agrobacterium sp.* strain CP4, a common soil-borne bacterium, has been sequenced and shown to encode a 47.6 kDa EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). In plants, the EPSPS enzyme is located within the chloroplast. Thus, in the construction of PV-GHGT35, a chloroplast transit peptide coding sequence, *ctp2,* was joined to the *cp4 epsps* coding sequence to provide transport to the cotton chloroplast. The CP4 EPSPS protein produced in Roundup Ready plants is functionally identical to endogenous plant EPSPS enzymes with the exception that CP4 EPSPS naturally displays reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPSs (Padgette et al*.,* 1996). In conventionally bred plants, glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of 5-enolpyruvylshikimate-3-phosphate, thereby depriving plants of essential amino acids and secondary metabolites (Steinrucken and Amrhein, 1980; Haslam, 1993). In Roundup Ready plants, which have been improved through biotechnology to be tolerant to Roundup agricultural herbicides (i.e., exhibiting tolerance to Roundup agricultural herbicides due to the presence of CP4 EPSPS), aromatic amino acids and other metabolites that are necessary

for normal growth and development are produced by the continued action of the glyphosate-tolerant CP4 EPSPS enzyme (Padgette et al., 1996).

IV.C. The *Arabidopsis thaliana* EPSPS Transit Peptide (CTP2)

Within the expression cassettes, the *cp4 epsps* coding sequence is joined to a chloroplast transit peptide sequence, designated *ctp2*, derived from the *Arabidopsis thaliana epsps* gene (Klee and Rogers, 1987). This transit peptide directs the transport of the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Klee and Rogers, 1987; Kishore et al., 1988). Transit peptides are typically cleaved from the mature protein following delivery to the plastid (Della-Cioppa et al., 1986). The *ctp2* present in PV-GHGT35 is the same *ctp2* transit peptide sequence used in the development of the existing cotton product, Roundup Ready cotton.

IV.D. Regulatory Sequences

Starting from the right border region of plasmid PV-GHGT35, the *ctp2/cp4 epsps* coding sequence in the first gene expression cassette is under the regulation of the P-FMV/TSF1 transcriptional promoter. P-FMV/TSF1 is a chimeric promoter containing the *Arabidopsis thaliana* TSF1 gene promoter (encoding elongation factor EF-1 alpha, Axelos et al., 1989) and enhancer sequences from the figwort mosaic virus 35S promoter (Richins et al., 1987). Located between the P-FMV/TSF1 promoter and the *ctp2/cp4 epsps* coding sequence is the nontranslated L-TSF1 leader sequence (exon 1) from the *A. thaliana* TSF1 gene and the I-TSF1 nontranslated intron from the *A. thaliana* TSF1 gene (Axelos et al., 1989). The *ctp2/cp4 epsps* coding sequence is linked at the 3' end to the T-E9 DNA sequence derived from *Pisum sativum*, containing the 3' nontranslated region of the pea ribulose-1,5-bisphosphate carboxylase (*rbc*), small subunit E9 gene (Coruzzi et al., 1984) for transcriptional termination and polyadenylation of the *cp4 epsps* mRNA.

Following tandem to the first gene expression cassette described above, the second *ctp2/cp4 epsps* gene expression cassette is under the regulation of the P-35S/ACT8 transcriptional promoter. P-35S/ACT8 is a chimeric promoter containing the promoter of the ACT8 gene of *A. thaliana* (An et al., 1996) combined with the enhancer sequences of the cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987). Located between the P-35S/ACT8 promoter and the *ctp2/cp4 epsps* coding sequence is the nontranslated leader sequence L-ACT8 from the ACT8 gene of *A. thaliana* and the I-ACT8 intron and flanking exon sequence from the ACT8 gene of *A. thaliana* (An et al., 1996). The *ctp2/cp4 epsps* coding sequence is linked at the 3' end to the T-E9 DNA sequence (Coruzzi et al., 1984), identical to the first *cp4 epsps* gene expression cassette, for transcriptional termination and polyadenylation of the *cp4 epsps* mRNA.

IV.E. T-DNA Borders

Plasmid vector PV-GHGT35 contains border regions that delineate the T-DNA to be transferred into cotton and are necessary for the efficient transfer of the T-DNA into the plant cell. These are termed the right border and left border regions (Figures V-1a,b, Table IV-1). The right border region is comprised of sequences derived from *Agrobacterium* containing the right border (RB) sequences (Depicker et al., 1982). The left border region contains sequences derived from *Agrobacterium* containing the left border (LB) sequences (Barker et al., 1983).

IV.F. Genetic Elements Outside of the Borders

The elements described below are present on plasmid vector PV-GHGT35 (Figures V-1a, b), but exist outside the T-DNA borders. Hence, they were not expected to be transferred into the cotton genome, and their absence in MON 88913 has been confirmed by data presented in Section V. of this petition.

- *OR-ORI V:* Origin of replication for maintenance of plasmid in *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al., 1981).

- *CR-rop:* Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).

- *OR-ORI-PBR322:* Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978).

- *CR-aad:* Coding sequence for Tn7 adenylyltransferase conferring spectinomycin and streptomycin resistance (Fling et al., 1985).

Table IV-1. Summary of Genetic Elements in PV-GHGT35.

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	1-8	Intervening linker sequences
B[1]- Left Border Region	9-450	DNA sequence derived from *Agrobacterium* containing the left border (LB) sequence for the efficient transfer of the DNA (Barker et al., 1983).
Intervening Sequence	451-536	Intervening linker sequences
OR[2]-ORI V	537-1174	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al., 1981).
Intervening Sequence	1175-2329	Intervening linker sequences
CR[3]-rop	2330-2802	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989).
Intervening Sequence	2803-3050	Intervening linker sequences
OR–ORI-PBR322	3051-3679	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978).
Intervening Sequence	3680-4221	Intervening linker sequences
CR - *aad*	4222-5010	Coding sequence for Tn7 adenylyltransferase conferring spectinomycin and streptomycin resistance (Fling et al., 1985).
Intervening Sequence	5011-5204	Intervening linker sequences
B-Right Border Region	5205-5535	DNA sequences derived from *Agrobacterium* containing the right border (RB) sequence for the efficient transfer of the DNA (Depicker et al., 1982).
Intervening sequence	5536-5645	Intervening linker sequences
P[4]-FMV/TSF1	5646-6685	Chimeric promoter containing the *Arabidopsis thaliana tsf1* gene promoter (encoding elongation factor EF-1alpha [Axelos, et al., 1989]) and enhancer sequences from the Figwort Mosaic virus 35S promoter (Richins et al., 1987).
L[5]-TSF1	6686-6731	Leader (exon 1) from the *Arabidopsis thaliana tsf1* gene encoding elongation factor EF-1alpha (Axelos et al., 1989).
I[6]-TSF1	6732-7353	Intron from the *Arabidopsis thaliana tsf1* gene encoding elongation factor EF-1alpha (Axelos et al., 1989).
Intervening Sequence	7354-7362	Intervening linker sequences

[1] B - Border
[2] OR - Origin of replication
[3] CR – Coding region
[4] P - Promoter
[5] L - Leader
[6] I - Intron

Table IV-1 (Continued). Summary of Genetic Elements in PV-GHGT35.

Genetic Element	Location in Plasmid	Function (References)
TS[7]- *ctp2*	7363-7590	DNA sequences derived from *Arabidopsis thaliana*. Chloroplast transit peptide, derived from the *Arabidopsis thaliana epsps* gene, present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis (Klee and Rogers, 1987).
cp4 epsps	7591-8958	DNA sequence containing synthetic coding sequence for the CP4 EPSPS protein from *Agrobacterium sp.* strain CP4 (Padgette et al., 1996; Barry et al., 1997).
Intervening Sequence	8959-9000	Intervening linker sequences
T[8]-E9	9001-9643	DNA sequences derived from *Pisum sativum,* containing the 3' nontranslated region of the pea ribulose-1, 5-bisphosphate carboxylase (*rbc*), small subunit E9 gene (Coruzzi et al., 1984).
Intervening sequence	9644-9681	Intervening linker sequences
P-35S/ACT8	9682-10856	Chimeric promoter containing the promoter of the *act8* gene of *Arabidopsis thaliana* (An et al., 1996) combined with the enhancer sequences of the Cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987).
L-ACT8	10857-10997	Leader sequence from the *act8* gene of *Arabidopsis thaliana* (An et al., 1996).
I-ACT8	10998-11470	Intron and flanking exon sequence from the *act8* gene of *Arabidopsis thaliana* (An et al., 1996).
Intervening Sequence	11471-11478	Intervening linker sequences
TS-*ctp2*	11479-11706	DNA sequences derived from *Arabidopsis thaliana*. Chloroplast transit peptide, derived from the *Arabidopsis thaliana epsps* gene, present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis (Klee and Rogers, 1987).
CR - *cp4 epsps*	11707-13074	DNA sequence containing synthetic coding sequence for the CP4 EPSPS protein from *Agrobacterium sp.* strain CP4 (Padgette et al., 1996; Barry et al., 1997).
Intervening Sequence	13075-13080	Intervening linker sequences
T-E9	13081-13723	DNA sequences derived from *Pisum sativum,* containing the 3' nontranslated region of the pea ribulose-1, 5-bisphosphate carboxylase (*rbc*), small subunit E9 gene (Coruzzi et al., 1984).
Intervening Sequence	13724-13741	Intervening linker sequences

[7] TS - Targeting sequence
[8] T - 3' untranslated transcriptional termination sequence and polyadenylation signal sequences

IV.G. Deduced Amino Acid Sequence

The amino acid sequence of the plant-produced CP4 EPSPS protein in MON 88913 was deduced from the full-length *cp4 epsps* coding sequence present in PV-GHGT35 and in MON 88913 (Figure IV-1).

```
  1     MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
 51     LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101     TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151     RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201     DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251     PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301     VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351     RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401     HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451     DTKAA
```

Figure IV-1. Deduced Amino Acid Sequence for the CP4 EPSPS Protein Produced in MON 88913.

V. Genetic Analysis

Molecular analysis was performed to characterize the DNA insert in MON 88913. This analysis demonstrated that MON 88913 contains a single, intact insert comprised of two *cp4 epsps* gene expression cassettes of the T-DNA of plasmid PV-GHGT35: (1) the *ctp2/cp4 epsps* coding sequence whose transcription is directed by the FMV/TSF1 chimeric promoter, the leader (exon 1) and intron sequences from the *Arabidopsis thaliana tsf1* gene, and the transcriptional termination and polyadenylation sequence derived from the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1, 5-bisphosphate carboxylase (*rbc*) small subunit E9 gene; (2) a second *ctp2/cp4 epsps* coding sequence, identical to the first, whose transcription is directed by the 35S/ACT8 chimeric promoter, the leader, intron and flanking sequences from the *act8* gene of *Arabidopsis thaliana,* and the transcriptional termination and polyadenylation sequence derived from the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1, 5-bisphosphate carboxylase (*rbc*) small subunit E9 gene. This T-DNA was inserted into the cotton genome and results in the synthesis of a homogeneous CP4 EPSPS protein from the two *cp4 epsps* gene expression cassettes. The *ctp2* chloroplast transit peptide sequence, derived from the *Arabidopsis thaliana epsps* gene, is present to direct the CP4 EPSPS protein to the cotton chloroplast.

V.A. DNA Insert Characterization

This section details the molecular analyses that were performed to characterize the integrated DNA insert in MON 88913, verify the DNA insert junction with the cotton genome, and verify the insert stability across generations.

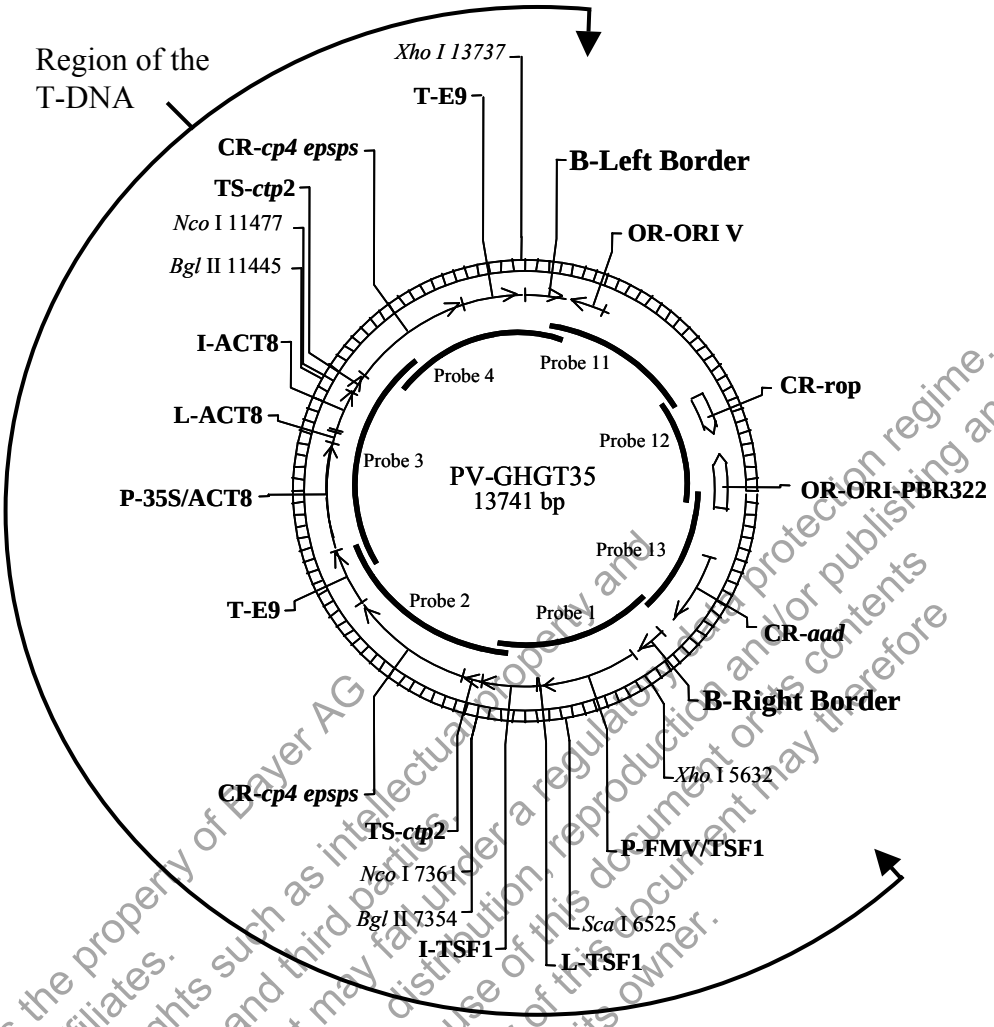
Genomic DNA from MON 88913 was digested with restriction enzymes and subjected to Southern blot analyses to characterize the DNA that was integrated into the cotton genome. A map of plasmid vector PV-GHGT35 annotated with the probes used in the Southern analyses is presented in Figures V-1a and V-1b. A linear map depicting restriction sites within the DNA insert, as well as within the cotton genomic DNA flanking the insert is shown in Figure V-2. The materials and methods used in the analyses are presented in Appendix B.

<u>*Insert and Copy Number*</u>

The insert number (the number of integration sites of the T-DNA in the cotton genome) was evaluated by digesting DNA of MON 88913 and MON 88913(-) with the restriction enzyme *Spe* I that does not cleave within the T-DNA. This enzyme should release a restriction fragment containing the entire DNA insert and adjacent plant genomic DNA (Figure V-2). The number of restriction fragments detected indicates the number of inserts present in MON 88913. The number of copies of the T-DNA integrated at a single locus was determined by digesting MON 88913 DNA with the combination of restriction enzymes *Spe* I and *Sca* I. *Spe* I alone should release a restriction fragment containing the DNA insert and adjacent plant genomic DNA, while the *Sca* I cleaves once within the DNA insert (Figure V-2). If MON 88913 contains one copy of the T-DNA, probing with the T-DNA will result in two bands, each band representing a portion

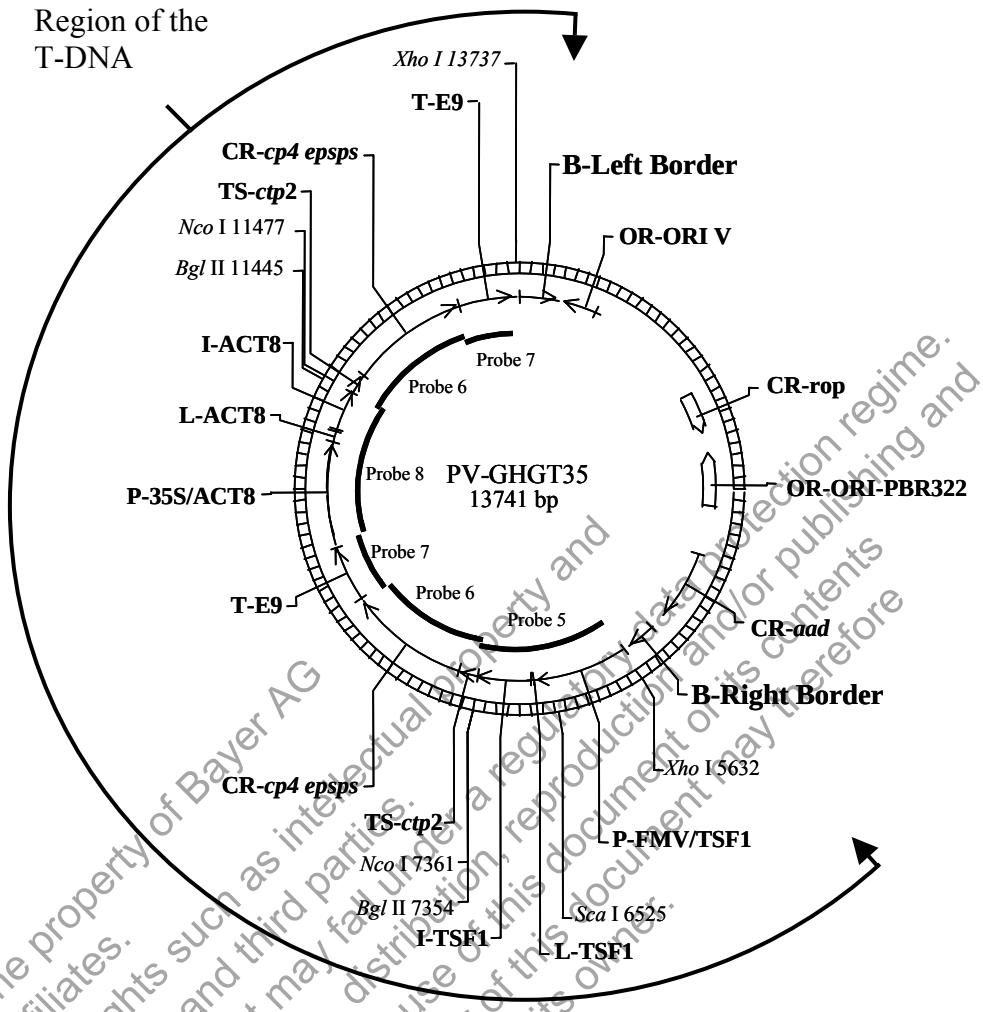
of the DNA insert along with adjacent plant genomic DNA. The blot was examined with four overlapping radiolabeled probes (probes 1 – 4, Figure V-1a) that spanned the entire T-DNA. The results of this analysis are presented in Figure V-3. For estimating the sizes of bands present in the long-run lanes of Southern blots, the molecular weight markers on the left side of the figure were used. For estimating the sizes of bands present in the short-run lanes, the molecular weight markers on the right side of the figure were used. The concept of using both long and short gel electrophoresis run times (runs) for the Southern blots was to assist in elucidating closely migrating DNA restriction fragments and to ensure that small molecular weight fragments were retained at the bottom of the agarose gel. Long runs provide enhanced resolution for higher molecular weight restriction fragments, and short runs provide retention and resolution of smaller molecular weight restriction fragments.

Plasmid PV-GHGT35 DNA digested with *Nco* I mixed with MON 88913(-) DNA digested with *Spe* I (lanes 7 and 8) produced the expected size bands of ~9.6 kb and 4.1 kb (Figure V-3). MON 88913 DNA digested with *Spe* I (lanes 3 and 9) produced a single band of ~13.0 kb. This result indicates that MON 88913 contains one DNA insert located on an ~13.0 kb *Spe* I restriction fragment. MON 88913 DNA digested with a combination of *Spe* I and *Sca* I (lanes 4 and 10) produced two unique bands at ~12.0 kb and ~1.2 kb in lane 10, representing the expected two border fragments that indicate only a single copy of DNA insert is present. The ~1.2 kb band expected in lane 4 (long run) ran off the gel and is not visible in the figure. MON 88913(-) DNA digested with *Spe* I alone (lanes 1 and 5) or a combination of *Spe* I and *Sca* I (lanes 2 and 6) produced no hybridization signal (Figure V-3). The faint mark observed at ~40 kb in lane 4 is a nonspecific hybridization artifact. Because this appears only in lane 4 of the long run and not in lane 10 of the short run and does not obscure any expected hybridization signals, it does not affect the interpretation of this Southern blot.



Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
1	T-DNA Probe 1	5521	8049	2.5
2	T-DNA Probe 2	7324	9829	2.5
3	T-DNA Probe 3	9518	12024	2.5
4	T-DNA Probe 4	294	11673	2.4
11	Backbone Probe 1	276	2069	1.8
12	Backbone Probe 2	1976	4109	2.1
13	Backbone Probe 3	4019	5525	1.5

Figure V-1a. Plasmid Vector PV-GHGT35 and Plasmid Backbone Probes.
Circular map of the plasmid vector PV-GHGT35 containing the T-DNA used via *Agrobacterium*-mediated transformation to create MON 88913. Four overlapping probes corresponding to the T-DNA and three overlapping probes corresponding to the backbone are drawn on the interior of the map. Genetic elements and restriction sites for enzymes used in the Southern analysis (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. Probes used in the Southern analysis are detailed in the accompanying list.



Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
5	P-FMV/TSF1 + L-TSF1/I-TSF1 Probe	7350	5633	1.7
6	TS-*ctp2*/CR-*cp4 epsps* Probe	7361	8958	1.6
7	T-E9 Probe	9001	9643	0.6
8	P-35S/ACT8 + L-ACT8/I-ACT8 Probe	9672	11469	1.8
6	TS-*ctp2*/CR-*cp4 epsps* Probe	11477	13074	1.6
7	T-E9 Probe	13081	13723	0.6

Figure V-1b. Plasmid Vector PV-GHGT35 and Individual Element Probes.
Circular map of the plasmid vector PV-GHGT35 containing the T-DNA used via *Agrobacterium*-mediated transformation to create MON 88913. Probes corresponding to each of the elements are drawn on the interior of the map. Genetic elements and restriction sites for enzymes used in the Southern analysis (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. Probes used in the Southern analysis are detailed in the accompanying list. Probes six and seven each hybridize to two different sections of the T-DNA.

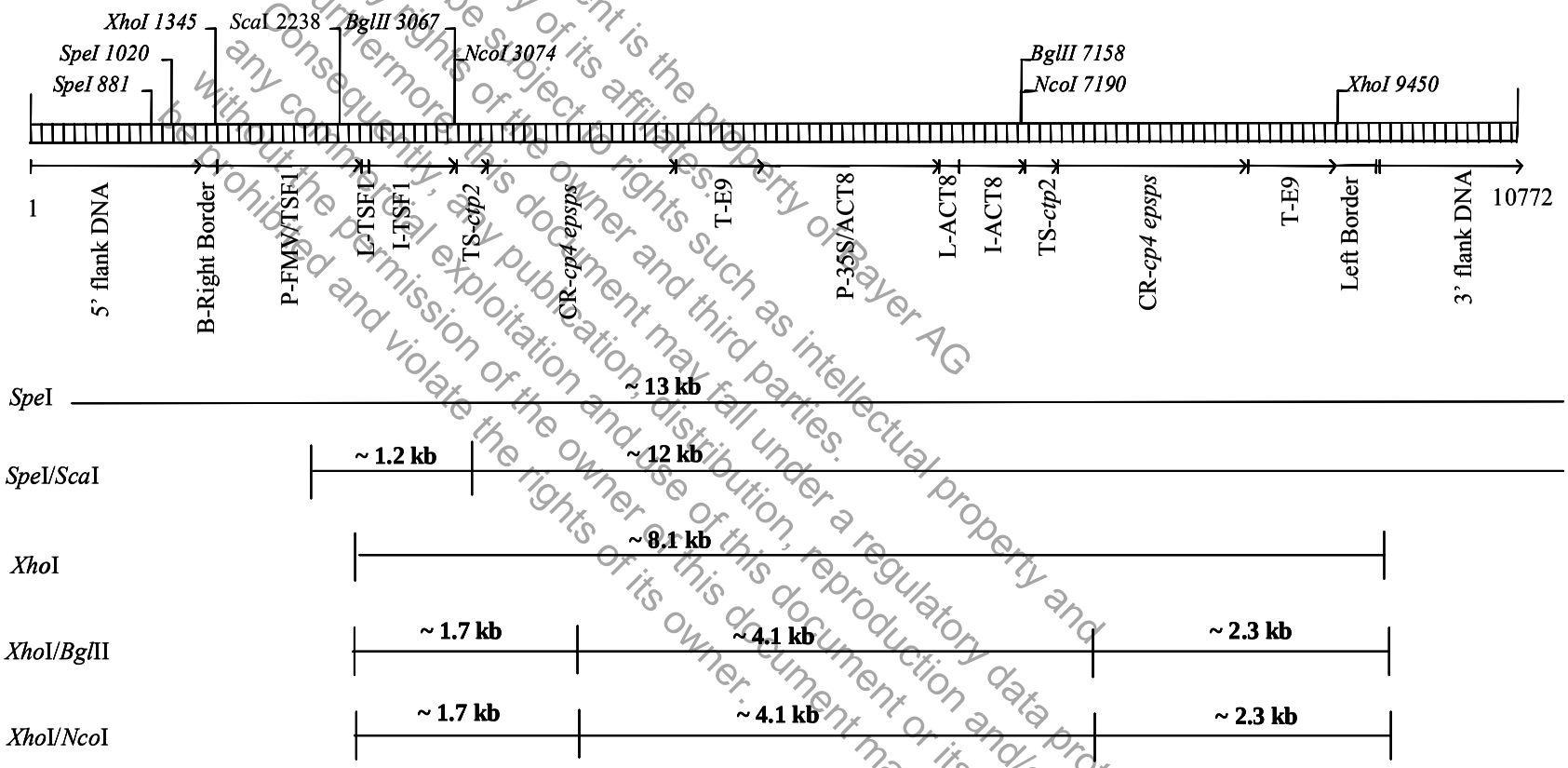


Figure V-2. Map of the DNA Insert in MON 88913.
A linear map of the DNA insert and adjacent DNA flanking the insert in MON 88913 is shown. Identified on the map are genetic elements within the DNA insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analysis. Arrows indicate the direction of transcription. MON 88913 contains one copy of the DNA insert at a single integration locus, so this schematic is also a representation of the T-DNA of PV-GHGT35. However, intact left and right borders are not implied. The expected size of the full DNA insert upon digestion with *Xho* I is ~8.1 kb. Base pairs 1232 – 9743 represent the inserted DNA corresponding to base pairs 5518 – 288 from PV-GHGT35.

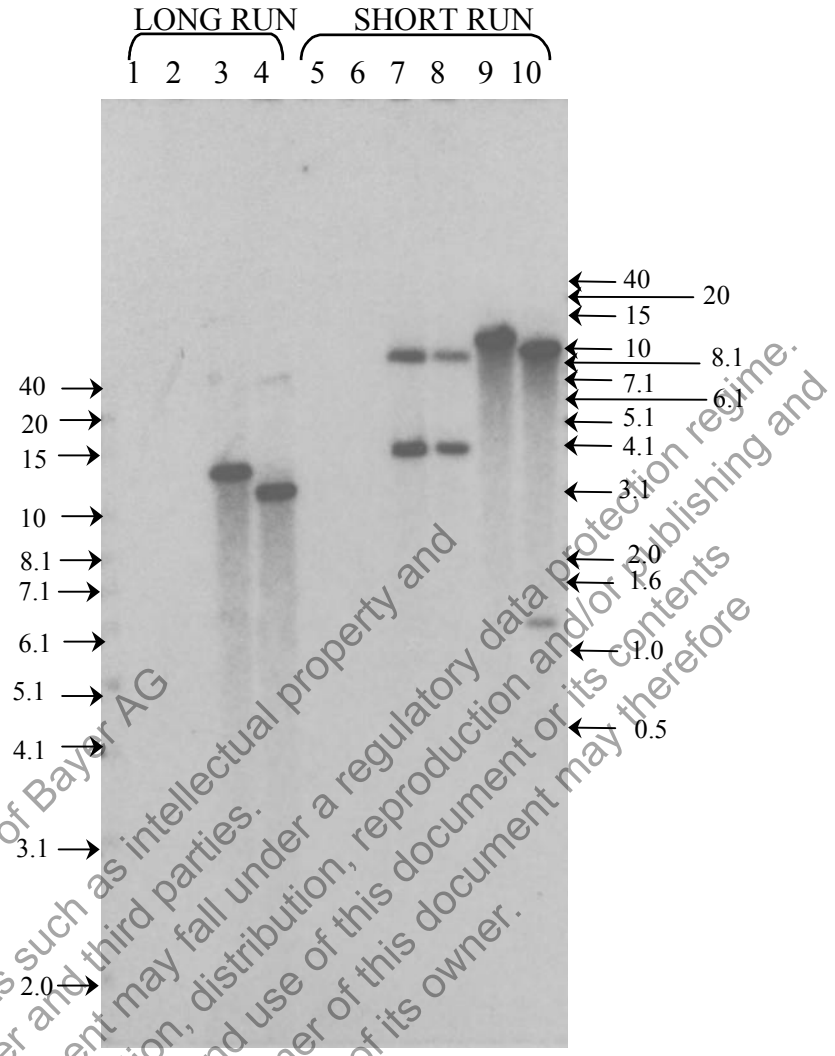


Figure V-3. Southern Blot Analysis of MON 88913: Insert and Copy Number Analyses.

The blot was probed simultaneously with four ^{32}P-labeled probes that spanned the entire length of the T-DNA (probes 1, 2, 3, and 4, Figure V-1a). Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

Lane 1: MON 88913(-) (*Spe* I)
2: MON 88913(-) (*Spe* I and *Sca* I)
3: MON 88913 (*Spe* I)
4: MON 88913 (*Spe* I and *Sca* I)
5: MON 88913(-) (*Spe* I)
6: MON 88913(-) (*Spe* I and *Sca* I)
7: MON 88913(-) (*Spe* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Spe* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (*Spe* I)
10: MON 88913 (*Spe* I and *Sca* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

Confirmation of the Absence of Plasmid PV-GHGT35 Backbone

MON 88913 and MON 88913(-) DNA were digested with either *Spe* I or a combination of *Spe* I and *Sca* I. Plasmid PV-GHGT35 DNA digested with *Nco* I was mixed with control genomic DNA digested with *Spe* I and then loaded on the gel to serve as a positive hybridization control. The blot was examined simultaneously with three overlapping probes (probes 11, 12, and 13, Figure V-1a) that span the backbone (sequences outside of the T-DNA) present in PV-GHGT35. The backbone probes were expected to cross-hybridize with the molecular weight markers because of common genetic elements. Therefore, these lanes were removed from the blot prior to hybridization. Aligning these lanes to the blot after hybridization allowed for appropriate annotation of the molecular weight markers on the film. MON 88913(-) DNA digested with *Spe* I (lanes 1 and 5) or a combination of *Spe* I and *Sca* I (lanes 2 and 6) showed no detectable hybridization bands, as expected for MON 88913(-) (Figure V-4). Plasmid PV-GHGT35 *Nco* I restriction fragments mixed with MON 889139(-) DNA digested with *Spe* I (lanes 7 and 8) produced one expected size band at ~9.6 kb. MON 88913 DNA digested with either *Spe* I (lanes 3 and 9) or a combination of *Spe* I and *Sca* I (lanes 4 and 10) showed no detectable hybridization signal, indicating that MON 88913 does not contain any detectable backbone sequence from the transformation vector PV-GHGT35.

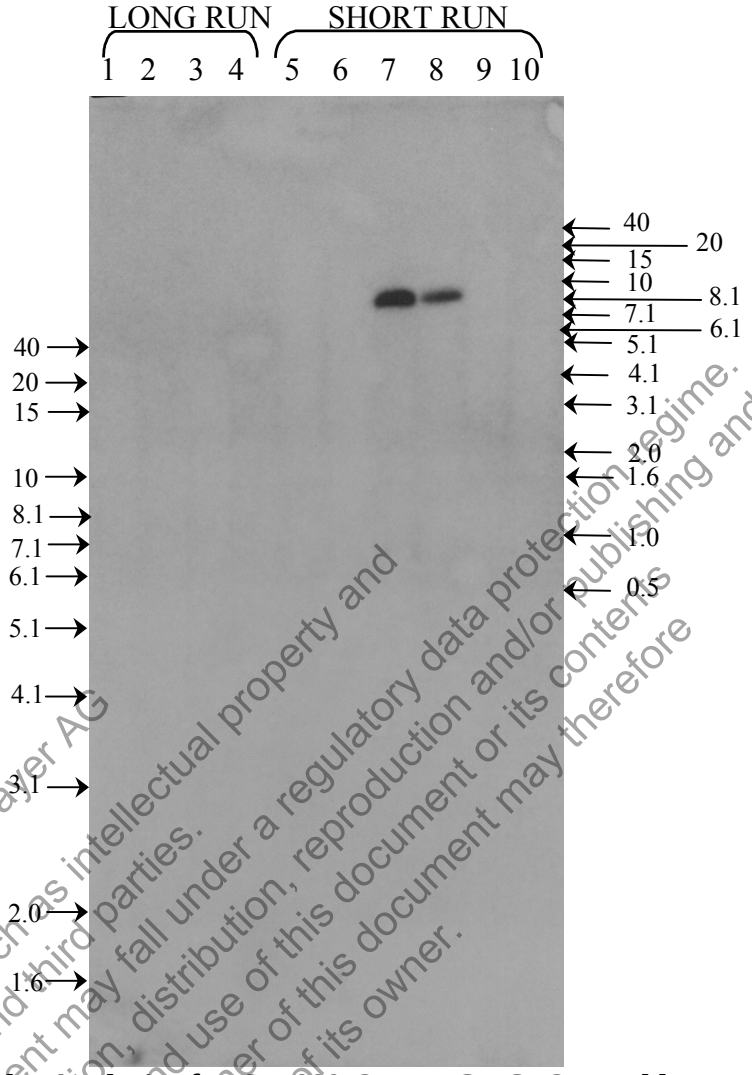


Figure V-4. Southern Blot Analysis of MON 88913: PV-GHGT35 Backbone Analysis.

Each blot was probed simultaneously with three ^{32}P-labeled probes that span the entire backbone sequence (probes 11, 12, and 13, Figure V-1a) of plasmid PV-GHGT35. Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

Lane 1: MON 88913(-) (*Spe* I)
2: MON 88913(-) (*Spe* I and *Sca* I)
3: MON 88913 (*Spe* I)
4: MON 88913 (*Spe* I and *Sca* I)
5: MON 88913(-) (*Spe* I)
6: MON 88913(-) (*Spe* I and *Sca* I)
7: MON 88913(-) (*Spe* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Spe* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (*Spe* I)
10: MON 88913 (*Spe* I and *Sca* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

CP4 EPSPS Expression Cassette Integrity

The integrity of the two inserted *cp4 epsps* gene expression cassettes and their associated genetic elements was assessed by digestion of the MON 88913 DNA with the restriction enzyme *Xho* I, or the combination of restriction enzymes *Xho* I and *Bgl* II, or the combination of restriction enzymes *Xho* I and *Nco* I. Digestion with *Xho* I generates a single ~8.1 kb restriction fragment containing both expression cassettes of the entire T-DNA (Figure V-2). Digestion of MON 88913 DNA with the combination of *Xho* I and *Bgl* II when examined with the P-FMV/TSF1 + L-TSF1/I-TSF1 probe was expected to generate a single restriction fragment of ~1.7 kb containing the P-FMV/TSF1 promoter, the L-TSF1 leader, and the I-TSF1 intron. Digestion of the MON 88913 DNA with the combination of *Xho* I and *Nco* I was expected to generate two restriction fragments of ~4.1 kb and ~2.3 kb when examined with the TS-*ctp2/cp4 epsps* probe (Figure V-2). Digestion MON 88913 DNA with the combination of *Xho* I and *Nco* I was expected to generate a single restriction fragment of ~4.1 kb when examined with the P-35S/ACT8 + L-ACT8/I-ACT8 probe containing the P-35S/ACT8 promoter, L-ACT8 leader, and I-ACT8 intron (Figure V-2). Plasmid PV-GHGT35 DNA previously digested with *Nco* I was mixed with MON 88913(-) DNA digested with *Xho* I and then loaded on the gel to serve as a positive hybridization control. The individual Southern blots were probed with P-FMV/TSF1 + L-TSF1/I-TSF1, TS-*ctp2*/*cp4 epsps* coding region, T-E9, or P-35S/ACT8 + L-ACT8/I-ACT8 (probes 5, 6, 7, and 8 respectively; Figure V-1b). Because the TS–*ctp2*/*cp4 epsps* coding region and T-E9 are identical in both cassettes, the same banding pattern is expected to be produced with each of these probes for the two *cp4 epsps* gene expression cassettes.

P-FMV/TSF1 + L-TSF1/I-TSF1

When examined with the P-FMV/TSF1 + L-TSF1/I-TSF1 probe (probe 5, Figure V-1b), plasmid PV-GHGT35 DNA previously digested with *Nco* I and mixed with MON 88913(-) DNA digested with *Xho* I (lanes 7 and 8) produced the expected size band at ~9.6 kb. The results are shown in Figure V-5. The probe was expected to cross-hybridize with the molecular weight marker bands because of common genetic elements. Therefore, these lanes were removed from the blot prior to hybridization. Aligning these lanes to the corresponding blot after hybridization allowed for appropriate annotation of the molecular weight markers on the film. MON 88913 DNA digested with *Xho* I (lanes 3 and 9) produced the expected band of ~8.1 kb. MON 88913 DNA digested with the combination of *Xho* I and *Bgl* II (lanes 4 and 10) produced a single predicted size band of ~1.7 kb. MON 88913(-) DNA digested with *Xho* I (lanes 1 and 5), or the combination of *Xho* I and *Bgl* II (lanes 2 and 6) showed no detectable hybridizing bands, as expected. Thus, based on the results presented in Figure V-5, no unexpected bands were detected in the MON 88913 DNA, indicating that MON 88913 contains no additional, detectable P-FMV/TSF1 + L-TSF1/I-TSF1 elements other than those associated with the intact *cp4 epsps* gene expression cassette.

TS-ctp2/cp4 epsps

Southern blot analysis was performed using the TS-*ctp2*/*cp4 epsps* probe (probe 6, Figure V-1b), and the results are shown in Figure V-6. Plasmid PV-GHGT35 DNA previously digested with *Nco* I mixed with MON 88913(-) DNA digested with *Xho* I (lanes 7 and 8)

produced the expected size bands at ~9.6 kb and 4.1 kb. MON 88913 DNA digested with *Xho* I (lanes 3 and 9) produced the expected size band of ~8.1 kb. MON 88913 DNA digested with a combination of *Xho* I and *Bgl* II (lanes 4 and 10) produced the expected size bands of ~4.1 kb and 2.3 kb. MON 88913(-) DNA digested with *Xho* I (lanes 1 and 5) or a combination of *Xho* I and *Bgl* II (lanes 2 and 6) showed no detectable hybridizing bands, as expected. The migration of the ~8.1 kb *Xho* I fragment containing the entire DNA insert is slightly lower than indicated by the molecular weight marker band sizes. The migration of the ~4.1 kb plasmid fragment is slightly higher than indicated by the molecular weight marker band sizes. These slightly altered migrations may be because of the difference in salt concentrations between the MON 88913 DNA sample and the molecular weight marker (Sambrook and Russell, 2001). No unexpected bands were detected, indicating that MON 88913 contains no additional detectable TS-*ctp2*/*cp4 epsps* elements other than those associated with the intact *cp4 epsps* gene expression cassette. The aberrant signal observed at ~5.1 kb spanning lanes 5 and 6 is a background hybridization artifact and does not affect the interpretation of this Southern blot.

T-E9

Southern blot analysis was performed using the T-E9 probe (probe 7, Figure V-1b), and the results are shown in Figure V-7. Plasmid PV-GHGT35 DNA previously digested with *Nco* I mixed with MON 88913(-) DNA digested with *Xho* I (lanes 7 and 8) produced the expected size bands at ~9.6 kb and ~4.1 kb. MON 88913 DNA digested with *Xho* I (lanes 3 and 9) produced the expected size band of ~8.1 kb. MON 88913 DNA digested with a combination of *Xho* I and *Bgl* II (lanes 4 and 10) produced the expected size bands of ~4.1 kb and ~2.3 kb. MON 88913(-) DNA digested with *Xho* I (lanes 1 and 5) or a combination of *Xho* I and *Bgl* II (lanes 2 and 6) showed no detectable hybridization bands, as expected. No unexpected bands were detected, indicating that MON 88913 contains no detectable T-E9 elements other than those associated with the intact *cp4 epsps* gene expression cassettes.

P-35S/ACT8 + L-ACT8/I-ACT8

When examined with the P-35S/ACT8 + L-ACT8/I-ACT8 probe (probe 8, Figure V-1b), plasmid PV-GHGT35 DNA previously digested with *Nco* I and mixed with MON 88913(-) DNA digested with *Xho* I (lanes 7 and 8) produced one expected size band at ~4.1 kb. The results are shown in Figure V-8. The probe was expected to cross-hybridize with the molecular weight marker bands because of common genetic elements. Therefore, these lanes were removed from the blot prior to hybridization. Aligning these lanes to the corresponding blot after hybridization allowed for appropriate annotation of the molecular weight markers on the film. MON 88913 DNA digested with *Xho* I (lanes 3 and 9) produced the expected size band of ~8.1 kb. The migration of the ~8.1 kb *Xho* I fragment containing the entire DNA insert is slightly higher than indicated by the molecular weight marker band sizes. This slightly altered migration may be because of the difference in salt concentrations between the MON 88913 DNA sample and the molecular weight marker (Sambrook and Russell, 2001). MON 88913 DNA digested with a combination of *Xho* I and *Nco* I (lanes 4 and 10) produced the expected band of ~4.1 kb. MON 88913(-) DNA digested with *Xho* I (lanes 1 and 5), or a combination of *Xho* I and *Nco* I (lanes 2 and 6) showed no detectable hybridization bands, as expected.

No unexpected bands were detected, indicating that MON 88913 contains no additional, detectable P-35S/ACT8 + L-ACT8/I-ACT8 elements other than those associated with the intact *cp4 epsps* gene expression cassettes.

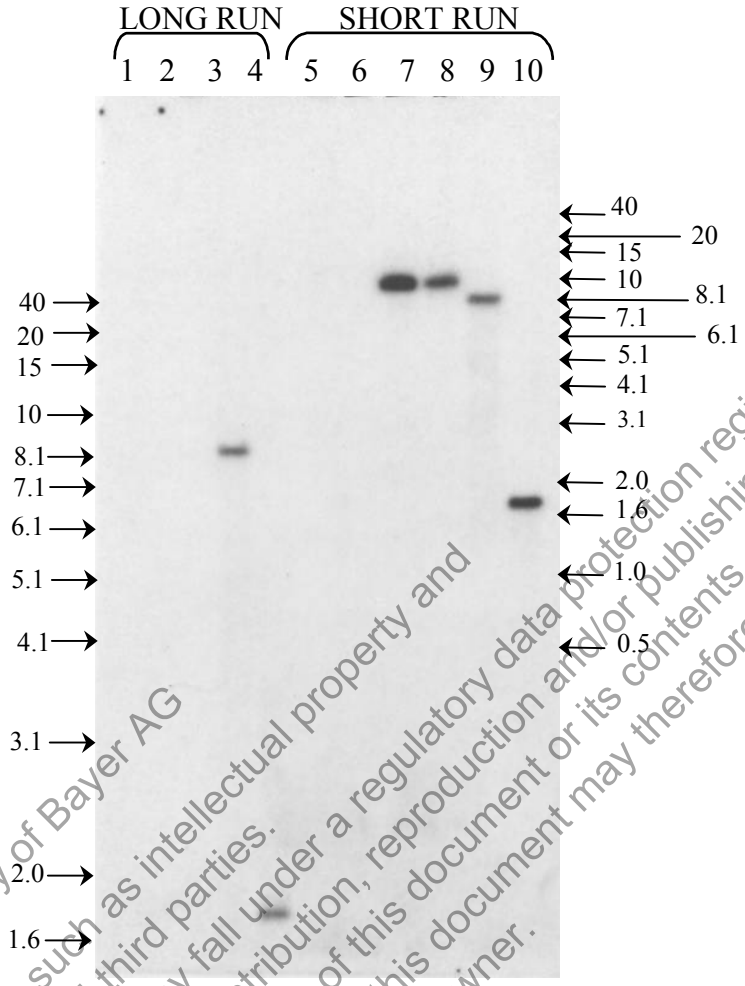


Figure V-5. Southern Blot Analysis of MON 88913: Insert Integrity Analysis with P-FMV/TSF1 + L-TSF1/I-TSF1 Probe.

The blot was probed with a ^{32}P-labeled probe that spanned the P-FMV/TSF1 promoter, L-TSF1 leader and I-TSF1 intron (probe 5, Figure V-1b). Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

Lane 1: MON 88913(-) (*Xho* I)
2: MON 88913(-) (*Xho* I and *Bgl* II)
3: MON 88913 (*Xho* I)
4: MON 88913 (*Xho* I and *Bgl* II)
5: MON 88913(-) (*Xho* I)
6: MON 88913(-) (*Xho* I and *Bgl* II)
7: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (*Xho* I)
10: MON 88913 (*Xho* I and *Bgl* II)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

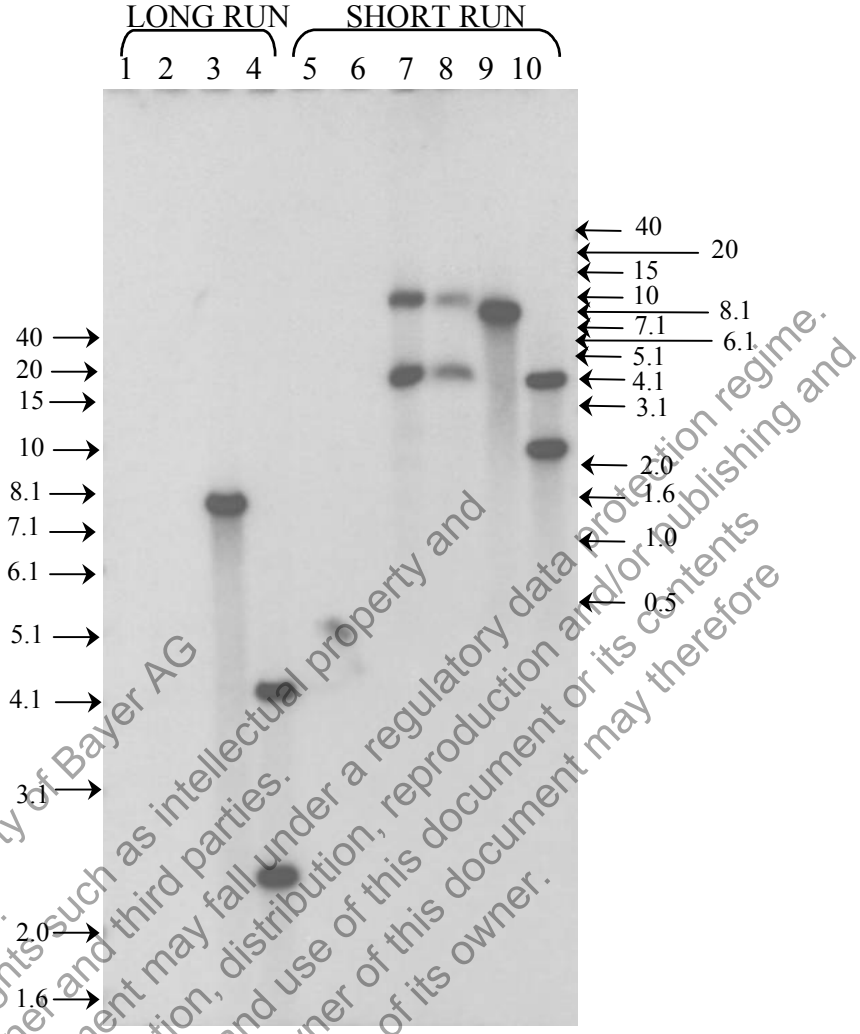


Figure V-6. Southern Blot Analysis of MON 88913: Insert Integrity Analysis with TS-*ctp2*/*cp4 epsps* Probe.

The blot was probed with a ^{32}P-labeled probe that spanned TS-*ctp2* (chloroplast transit peptide) and *cp4 epsps* (probe 6, Figure V-1b). Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

Lane 1: MON 88913(-) (*Xho*I)
2: MON 88913(-) (*Xho* I and *Bgl* II)
3: MON 88913 (*Xho* I)
4: MON 88913 (*Xho* I and *Bgl* II)
5: MON 88913(-) (*Xho* I)
6: MON 88913(-) (*Xho* I and *Bgl* II)
7: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (X*ho* I)
10: MON 88913 (*Xho* I and *Bgl* II)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

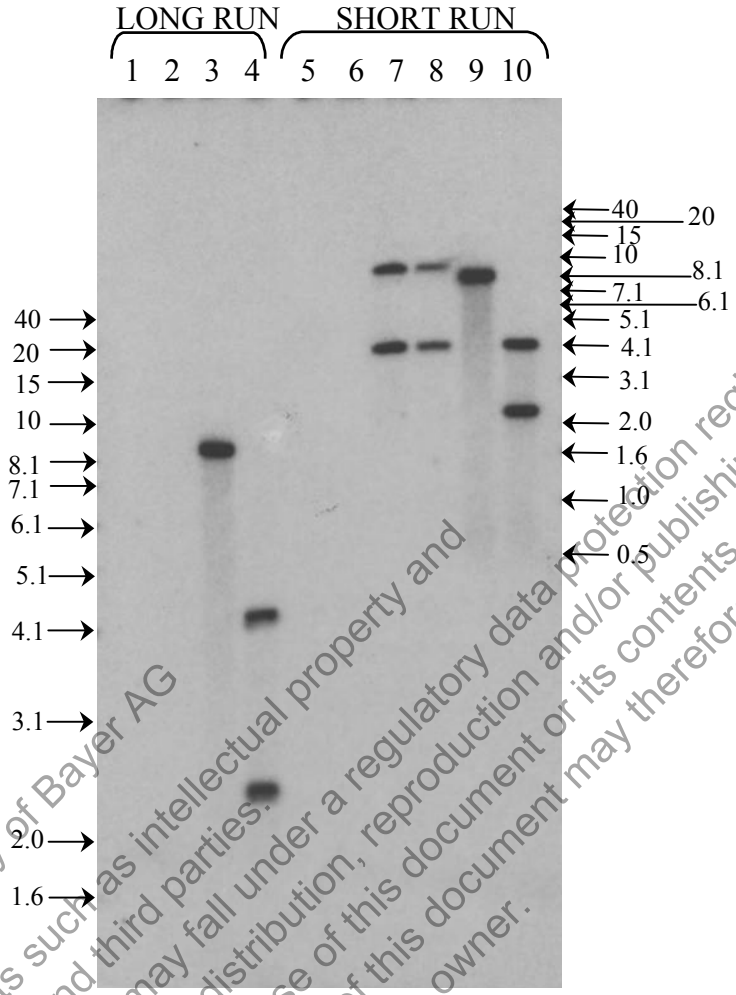


Figure V-7. Southern Blot Analysis of MON 88913: Insert Integrity Analysis with T-E9 Probe.

The blot was probed with a ^{32}P-labeled probe that spanned T-E9 (probe 7, Figure V-1b). Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

Lane 1: MON 88913(-) (*Xho*I)
2: MON 88913(-) (*Xho* I and *Bgl* II)
3: MON 88913 (*Xho* I)
4: MON 88913 (*Xho* I and *Bgl* II)
5: MON 88913(-) (*Xho* I)
6: MON 88913(-) (*Xho* I and *Bgl* II)
7: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (X*ho* I)
10: MON 88913 (*Xho* I and *Bgl* II)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

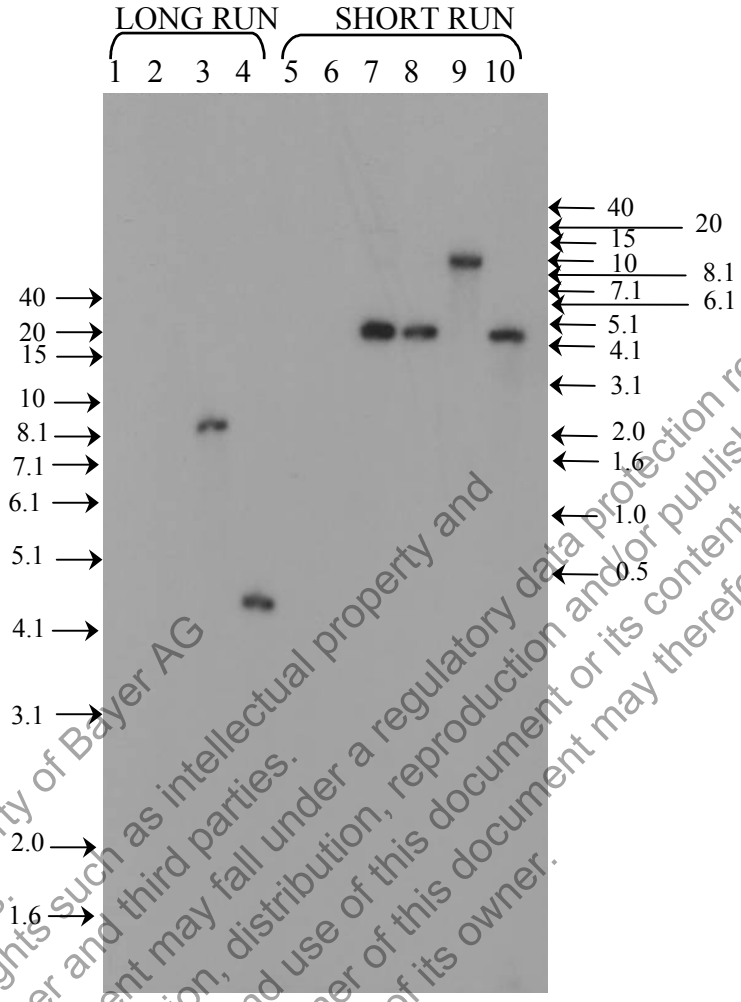


Figure V-8. Southern Blot Analysis of MON 88913: Insert Integrity Analysis with P-35S/ACT8 + L-ACT8/I-ACT8 Probe.

The blot was probed with a ^{32}P-labeled probe that spanned the P-35S/ACT8 promoter, L-ACT8 leader and I-ACT8 intron (probe 8, Figure V-1b). Each lane contains ~10 µg of digested genomic DNA isolated from seed. Lane designations are as follows:

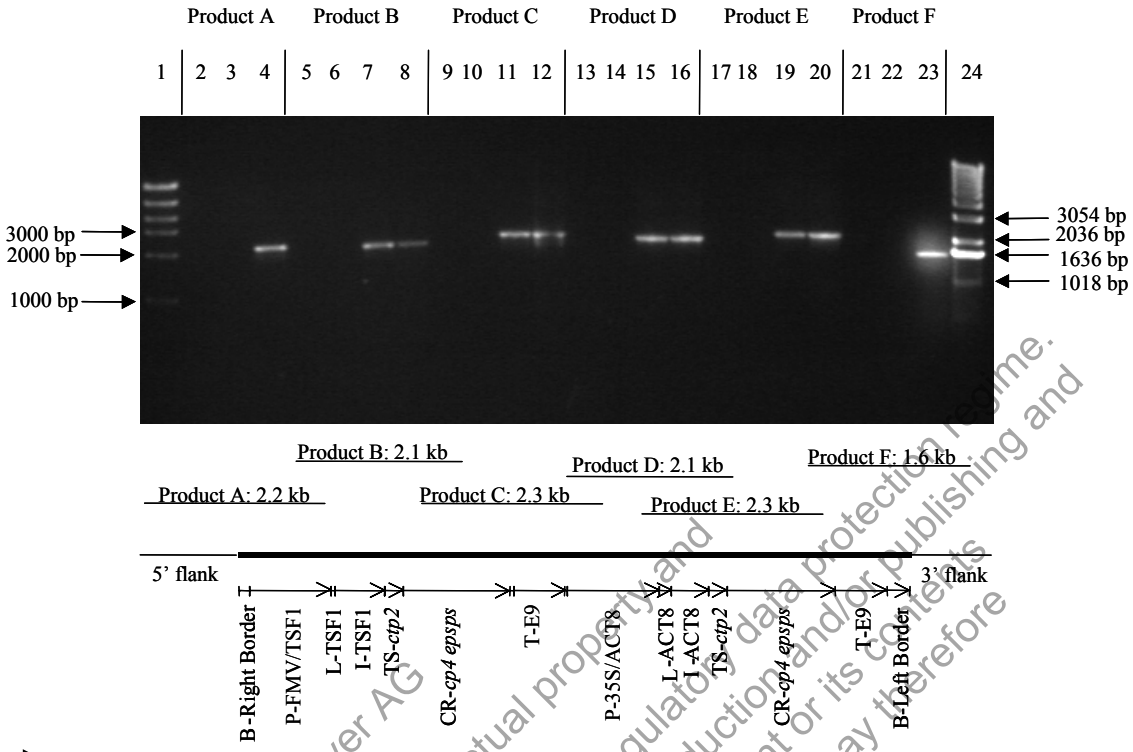
Lane 1: MON 88913(-) (*Xho* I)
2: MON 88913(-) (*Xho* I and *Nco* I)
3: MON 88913 (*Xho* I)
4: MON 88913 (*Xho* I and *Nco* I)
5: MON 88913(-) (*Xho* I)
6: MON 88913(-) (*Xho* I and *Nco* I)
7: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
8: MON 88913(-) (*Xho* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
9: MON 88913 (*Xho* I)
10: MON 88913 (*Xho* I and *Nco* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.B. Confirmation of the Organization of the DNA Insert and Verification of Adjacent Genomic DNA.

The organization of the elements within the DNA insert in MON 88913 was confirmed using PCR analysis by amplifying six overlapping regions of DNA that span the entire length of the insert and the immediate flanking cotton genomic DNA at the 5' and 3' junctions. The locations of the PCR products generated in relation to the insert, as well as the results of the PCR analyses, are shown in Figure V-9. The DNA sequence at the 5' and 3' ends of the insert were verified by PCR using cotton genomic DNA as a template. The PCR for the 5' insert-to-plant junction was performed using one primer designed to the 5' genomic flanking sequence, paired with a second primer in the 5' end of the DNA insert. The PCR for the 3' insert-to-plant junction was conducted using a primer designed to the 3' genomic flanking sequence, coupled with a second primer located in the 3' end of the DNA insert.

The control reactions containing no template DNA (lanes 2, 5, 9, 13, 17, and 21) did not generate PCR products with any of the primer sets, as expected. The MON 88913(-) reactions (lanes 3, 6, 10, 14, 18, and 22) also did not generate any PCR products, as expected. The plasmid PV-GHGT35 was used as a positive control in the four PCR analyses (Products B-E) that amplified products containing only the inserted DNA rather than the genomic DNA flanking the insert. In these four analyses, cotton genomic DNA from MON 88913, as well as the plasmid PV-GHGT35, generated the expected size PCR products of ~2.1 kb for Product B (lanes 7 and 8); ~2.3 kb for Product C (lanes 11 and 12); ~2.1 kb for Product D (lanes 15 and 16); and ~2.3 kb for Product E (lanes 19 and 20). MON 88913 DNA also generated the expected size PCR products of ~2.2 kb for Product A (lane 4) and ~1.6 kb for Product F (lane 23). The generation of the predicted size PCR products from MON 88913 establishes that the arrangement and linkage of elements in the insert are the same as those in plasmid PV-GHGT35 and that the elements within each *cp4 epsps* gene expression cassette are arranged as depicted in the schematic of the insert in Figure V-2.



→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

Figure V-9. Overlapping PCR Analysis Across the DNA Insert in MON 88913. PCR analyses demonstrating the linkage of the individual genetic elements within the DNA insert in MON 88913 were performed on MON 88913 genomic DNA extracted from seed. Lanes are marked to show which and how much product was loaded and is visualized on the agarose gel. The expected product size for each amplicon is highlighted in the illustration of the insert in MON 88913 that appears at the bottom of the figure.

Lane		Lane	
1:	Invitrogen High Mass DNA ladder	13:	No template (5 µl)
2:	No template (5 µl)	14:	MON 88913(-) negative segregant (5 µl)
3:	MON 88913(-) negative segregant (5 µl)	15:	MON 88913 genomic DNA (20 µl)
4:	MON 88913 genomic DNA (10 µl)	16:	PV-GHGT35 plasmid (3 µl)
5:	No template (5 µl)	17:	No template (5 µl)
6:	MON 88913(-) negative segregant (5 µl)	18:	MON 88913(-) negative segregant (5 µl)
7:	MON 88913 genomic DNA (5.5 µl)	19:	MON 88913 genomic DNA (9 µl)
8:	PV-GHGT35 plasmid (3 µl)	20:	PV-GHGT35 plasmid (3 µl)
9:	No template (5 µl)	21:	No template (5 µl)
10:	MON 88913(-) negative segregant (5 µl)	22:	MON 88913(-) negative segregant (5 µl)
11:	MON 88913 genomic DNA (3.5 µl)	23:	MON 88913 genomic DNA (12 µl)
12:	PV-GHGT35 plasmid (1 µl)	24:	Invitrogen 1Kb DNA ladder

V.C. Genetic Inheritance and Stability of the DNA Insert

Southern blot fingerprint analysis and progeny inheritance data were generated to establish the generational inheritance and stability of the DNA insert in MON 88913.

Southern Blot Analyses to Examine Insert Generational Stability
In order to demonstrate the stability of the DNA insert in MON 88913, Southern blot analysis was performed using DNA from multiple generations from the MON 88913 breeding tree. For reference, the breeding history of MON 88913 is presented in Figure III-2. The specific generations tested are indicated in the legends of Figures III-2 and V-10. For these analyses, MON 88913 and MON 88913(-) DNA samples were digested with the combination of restriction enzymes *Spe* I and *Sca* I. Digestion of MON 88913 with the combination of *Spe* I and *Sca* I produced two restriction fragments of ~12.0 kb and ~1.2 kb (lanes 4 – 8, Figure V-10). This is the same restriction pattern observed for the R3 generation shown in Figure V-3. Plasmid PV-GHGT35 DNA previously digested with *Nco* I and mixed with MON 88913(-) DNA digested with *Spe* I and *Sca* I produced the expected size bands of ~9.6 kb and ~4.1 kb for the positive hybridization control (lanes 2 and 3). The results of this analysis establish the stability of the DNA insert over the selected generations of MON 88913 representing multiple generations of the breeding tree.

Southern Blot Analyses to Confirm the Absence of Plasmid Backbone Across Multiple Generations
In addition to the DNA insert, generational stability analysis was conducted to confirm the absence of backbone sequence across sexual generations. Similar to the Southern analysis above, MON 88913 and MON 88913(-) DNA samples were digested with the combination of *Spe* I and *Sca* I. The blot was probed simultaneously with three radiolabeled probes that span the entire backbone sequence of plasmid PV-GHGT35 (probes 11, 12, and 13, Figure V-1a). Because of expected cross-hybridization of the probes to the molecular weight markers, these lanes were removed from each blot prior to hybridization. Aligning these lanes to the corresponding blot after hybridization allowed for appropriate annotation of the molecular weight markers on the film. Plasmid PV-GHGT35 DNA, previously digested with *Nco* I and mixed with MON 88913(-) genomic DNA, digested with *Spe* I and *Sca* I produced one expected size band of ~9.6 kb (lanes 2 and 3, Figure V-11). MON 88913(-) DNA digested with *Spe* I and *Sca* I (lane 1) produced no detectable hybridization bands, as expected. Also, the previously characterized R3 generation (Figure V-4) showed no detectable hybridization (lane 6, Figure V-11). Four additional generations of MON 88913 also showed no detectable hybridization (lanes 4, 5, 7, and 8, Figure V-11). These results indicate that none of the generations tested contain any detectable backbone sequence from the transformation vector PV-GHGT35.

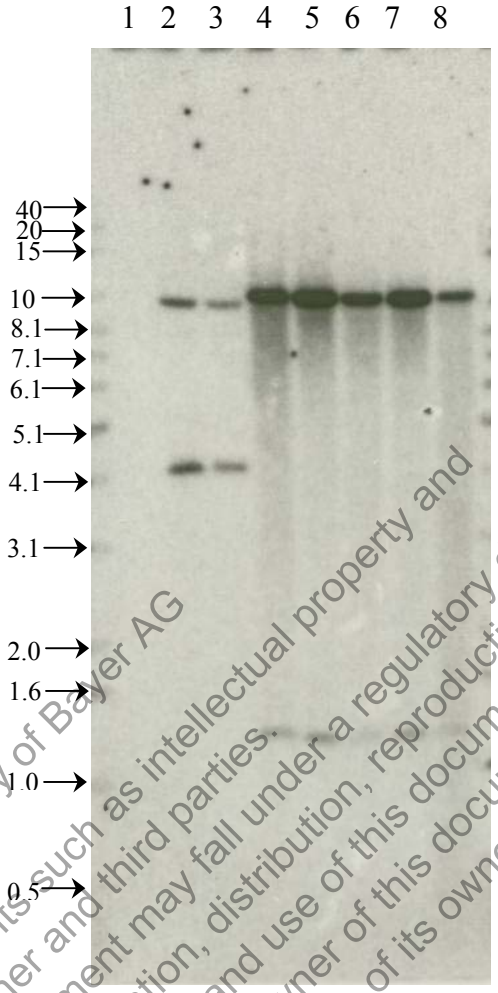


Figure V-10. Generational Stability of MON 88913: Insert and Copy Number Analysis.

The blot was probed simultaneously with four ^{32}P-labeled probes that span the T-DNA of PV-GHGT35 (probes 1, 2, 3 and 4, Figure V-1a). Each lane contains ~10 µg of digested genomic DNA isolated from seed or leaf material. The breeding history of MON 88913 is illustrated in Figure III-2. Lane designations are as follows:

Lane 1: MON 88913(-) (*Spe* I and *Sca* I)
2: MON 88913(-) (*Spe* I and *Sca* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
3: MON 88913(-) (*Spe* I and *Sca* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
4: MON 88913 - R1 (*Spe* I and *Sca* I)
5: MON 88913 - R2 (*Spe* I and *Sca* I)
6: MON 88913 - R3 (*Spe* I and *Sca* I)
7: MON 88913 - R4 (*Spe* I and *Sca* I)
8: MON 88913 - R5 (*Spe* I and *Sca* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

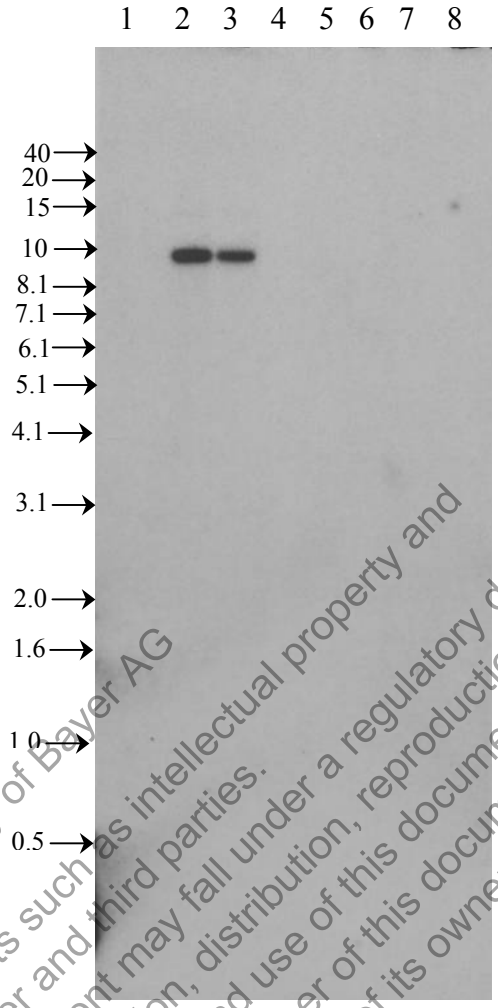


Figure V-11. Generational Stability of MON 88913: PV-GHGT35 Backbone Analysis.

The blot was probed simultaneously with three ^{32}P-labeled probes that span the entire backbone sequence (probes 11, 12, and 13, Figure V-1a) of plasmid PV-GHGT35. Each lane contains ~10 µg of digested genomic DNA isolated from seed or leaf material. Lane designations are as follows:

Lane 1: MON 88913(-) (*Spe* I and *Sca* I)
2: MON 88913(-) (*Spe* I and *Sca* I) spiked with PV-GHGT35 (*Nco* I) [1.0 copy]
3: MON 88913(-) (*Spe* I and *Sca* I) spiked with PV-GHGT35 (*Nco* I) [0.5 copy]
4: MON 88913 - R1 (*Spe* I and *Sca* I)
5: MON 88913 - R2 (*Spe* I and *Sca* I)
6: MON 88913 - R3 (*Spe* I and *Sca* I)
7: MON 88913 - R4 (*Spe* I and *Sca* I)
8: MON 88913 - R5 (*Spe* I and *Sca* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

Mendelian Segregation of Roundup Ready Flex Cotton

During the development of the MON 88913, analysis of phenotypic segregation data was conducted across several generations. A summary of results of these analyses is presented in Tables V-1, V-2, and V-3. The glyphosate tolerance of individual plants was determined by antibody strip-test for the CP4 EPSPS protein and/or tolerance to a Roundup agricultural herbicide spray. After self pollinating the MON 88913 plant regenerated from tissue culture, the R1 seeds were planted, and the resulting plants were expected to segregate in a 3:1 ratio in favor of the glyphosate tolerant phenotype as a single, dominant trait loci. In the R1 plants, the calculated Chi-Square value for phenotype was less than the critical value of 3.84 at the 5% level of error, and therefore MON 88913 demonstrated the expected 3:1 segregation in the R1 generation (Table V-1). The R2 generation represents a point in the breeding process where homozygous seed can be identified. Individual glyphosate-tolerant R1 plants were identified, self-pollinated to produce R2 seed, and then subjected to progeny screens to identify homozygous seed lots. Individual R2 families are expected to segregate 1:2 for homozygosity after glyphosate-sensitive individuals are removed from the population. Seventy-six R2 families were generated and tested for homozygosity. Chi-square analysis for homozygote recovery is presented in Table V-2. The calculated Chi-square value is less than the critical value of 3.84 at the 5% level of error. Therefore, the expected number of homozygous families were recovered during the breeding process. Selection of homozygous plant seed lots was successful in the R3 generation and was confirmed in generations R4 and R5. Homozygous MON 88913 seed lots are expected to segregate 1:0 for glyphosate-tolerance. Glyphosate-tolerance data from the R4 and R5 generations are summarized in Table V-3. These data confirm homozygosity and generational stability of MON 88913 and thus, the stability of the DNA insert.

Table V-1. Segregation Ratio for the MON 88913 Phenotype in the R1 Generation.

Generation	Phenotype	Expected Ratio	Expected No. of Plants(E)	Observed No. of Plants (O)	$(O-E)^2/E$
R1	Glyphosate tolerant	0.75	111.8	111	0.005
	Non-glyphosate tolerant	0.25	37.3	38	0.0151
		Total	149	149	0.0201

Critical value at 0.05 = 3.84; 1 degree of freedom.

Table V-2. Homozygous Recovery Ratio for the MON 88913 Phenotype in R2 Families.

Generation	Phenotype	Expected Ratio	Expected No. of Families (E)	Observed No. of Families(O)	$(O-E)^2/E$
R2	Homozygous	0.3333	25.3308	24	0.1310
	Segregating	0.6666	50.6616	52	0.0675
		Total	76	76	0.1985

Critical value at 0.05 = 3.84; 1 degree of freedom.

Table V-3. Confirmation of Homozygous Status in the R4 and R5 Generations.

Generation	Number Glyphosate Tolerant	Number Non-Glyphosate Tolerant	Test Method
R4	322	0	Roundup spray
R5	310	0	Roundup spray

V.D. Conclusions for Molecular Characterization

Molecular analyses were performed to characterize the integrated DNA insert in MON 88913. Southern blot genomic analyses were used to determine the DNA insert number (number of integration sites within the cotton genome), copy number (the number of copies within one insert), the intactness of the *cp4 epsps* gene expression cassettes, and to establish the absence of plasmid backbone sequences in the plant. The stability of the DNA insert across sexual generations was also demonstrated by Southern blot fingerprint. Polymerase chain reaction was performed to identify the 5' and 3' insert-to-genomic DNA junctions, and to confirm the organization of the elements within the DNA insert.

MON 88913 was generated by stably integrating two *cp4 epsps* gene expression cassettes into the cotton genome using *Agrobacterium*-mediated transformation. The data show that MON 88913 contains one copy of the DNA insert at a single integration locus on an ~13.0 kb *Spe* I restriction fragment that contains two intact *cp4 epsps* gene expression cassettes. No additional elements from the transformation vector PV-GHGT35, linked or unlinked to the intact DNA insert, were detected in the genome of MON 88913. Generational stability analysis demonstrated that the expected Southern blot fingerprint of MON 88913 has been maintained across five generations of breeding, thereby confirming the stability of the DNA insert over multiple generations and the absence of any detectable backbone sequence from plasmid PV-GHGT35. Finally, Mendelian segregation of the expected MON 88913 phenotype across multiple generations and families corroborates the molecular insert stability analysis and establishes the genetic behavior of the DNA insert as a single locus.

VI. Characterization of CP4 EPSPS

This section includes an evaluation of the CP4 EPSPS protein, establishes the equivalence of the CP4 EPSPS protein produced *in planta* to the CP4 EPSPS protein reference standard that was produced in *E. coli* and used in protein safety studies, assesses CP4 EPSPS production in MON 88913, and discusses the similarity of CP4 EPSPS to other EPSPSs with a history of safe use and environmental exposure.

VI.A. EPSPS Biochemistry and Mode of Action

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS: EC2.5.1.19) family of enzymes is ubiquitous to plants and microorganisms. EPSPS has been isolated from both sources, and its properties have been extensively studied (Harrison et al., 1996; Haslam, E., 1993; Klee and Rogers, 1987; Schonbrunn et al., 2001; Steinrüchen and Amrhein, 1984). The shikimate pathway, and hence the EPSPS protein, is absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings, 2001). The bacterial and plant enzymes are mono-functional with molecular mass of 44-48 kDa (Kishore et al., 1988). EPSPS proteins catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings, 2001). Because of the stringent specificity for substrates (i.e., EPSPS enzymes bind PEP and S3P), the only known metabolic product produced is EPSP, the penultimate product of the shikimic acid pathway. Shikimic acid is a substrate for the biosynthesis of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other aromatic molecules. It has been estimated that aromatic molecules, all of which are derived from shikimic acid, represent 35% or more of the dry weight of a plant (Franz et al., 1997).

MON 88913 contains the 5-enolpyruvylshikimate-3-phosphate synthase gene derived from *Agrobacterium sp*. strain CP4 (*cp4 epsps*). The *cp4 epsps* coding sequence encodes a 47.6 kDa EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Padgette et al., 1996). In conventional plants, glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of EPSP, thereby depriving plants of essential amino acids (Steinrucken and Amrhein, 1980; Haslam, 1993). In Roundup Ready plants, which are tolerant to Roundup agricultural herbicides, aromatic amino acids and other metabolites that are necessary for growth and development are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al., 1996).

Comparison of the kinetic parameters of CP4 EPSPS and endogenous plant EPSPS in the presence and absence of glyphosate elucidates the mechanism of glyphosate inhibition of EPSPS. Inhibition has been shown to proceed through the formation of a ternary

complex of EPSPS-S3P-glyphosate; the complex formation is ordered with glyphosate binding occurring only after the formation of a binary EPSPS-S3P complex. Glyphosate binding has been shown to be uncompetitive with respect to S3P and competitive with respect to PEP. The $K_{M(PEP)}$ for endogenous plant (*Petunia hybrida*) EPSPS and CP4 EPSPS are 5 µM and 12 µM, respectively (Franz et al., 1997). In contrast, the $K_{I(glyphosate)}$ for endogenous plant EPSPS (*Petunia hybrida*) and CP4 EPSPS has been found to be 0.4 µM and 2720 µM, respectively (Franz et al., 1997). This difference in glyphosate binding affinity is the basis for glyphosate tolerance in Roundup Ready crops that produce CP4 EPSPS.

VI.B. CP4 EPSPS Protein Levels in MON 88913 Tissues

The levels of CP4 EPSPS protein in MON 88913 were determined by a validated enzyme-linked immunosorbent assay (ELISA). Materials and methods for the ELISA analyses and validation are presented in Appendix B. The levels of CP4 EPSPS protein in young leaf, overseason leaf (OSL), root, seed, and pollen tissues were determined in tissues collected from MON 88913 produced in replicated field trials across four U.S. field locations during 2002 (Appendix C). CP4 EPSPS protein levels for all tissue types were calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was measured for young leaf, overseason leaf OSL-1, OSL-2, OSL-3; root; and seed tissues. Protein levels in these tissues were converted to a dry weight (dwt) basis by calculation. The mean CP4 EPSPS protein levels across four sites for young leaf, OSL1, OSL2, OSL3, root, and seed tissues of MON 88913 were 970, 1400, 690, 630, 99, and 340 µg/g dwt, respectively, (Table VI-1). The mean CP4 EPSPS protein level across four sites for pollen was 4.0 µg/g fwt. The levels of CP4 EPSPS protein in all tissue types from MON 88913(-) were less than the assay limits of quantitation (LOQ) presented in Table VI-1.

Table VI-1. CP4 EPSPS Protein Levels in MON 88913 Tissues.†

Tissue Type	Mean CP4 EPSPS Protein Level in µg/g fwt (SD)[1]	Range[2] (µg/g fwt)	Mean CP4 EPSPS Protein Level in µg/g dwt (SD)[3]	Range (µg/g dwt)	LOQ / LOD (µg/g fwt)
Young Leaf	170 (64)	64 – 260	970 (460)	270 – 1700	0.23 / 0.069
OSL1[4]	270 (99)	77 – 410	1400 (540)	480 – 2600	0.23 / 0.069
OSL2	170 (44)	63 – 260	690 (210)	290 – 1000	0.23 / 0.069
OSL3	160 (61)	66 – 260	630 (230)	290 – 1100	0.23 / 0.069
Root	31 (11)	19 – 64	99 (40)	57 – 200	0.23 / 0.073
Seed	310 (110)	67 – 550	340 (120)	72 – 580	2.7 / 1.7
Pollen	4.0 (0.22)	3.8 – 4.3	n/a[5]	n/a[5]	0.23 / 0.11

†Field-produced tissues in 2002 from Baldwin County, Alabama; Tulare County, California; Clarke County, Georgia; and Hockley County, Texas (Appendix C, Section C.3.).

[1]Protein levels are expressed as micrograms (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis. The arithmetic mean and standard deviation (SD) were calculated for each tissue type across sites.

[2]Minimum and maximum values were determined for each tissue type across all sites.

[3]Protein levels are expressed as µg/g of tissue on a dry weight (dwt) basis. The dwt values were calculated by dividing the fwt values by the dry weight conversion factors (Appendix B) obtained from moisture analysis data.

[4]Tissues OSL1 – OSL3 represent overseason leaves collected at different time points throughout the growing season (Appendix C).

[5]Because of limited quantities of cotton pollen, moisture levels could not be determined in this tissue and values are presented on a fwt basis only.

VI.C. Characterization of the CP4 EPSPS Protein Produced in MON 88913

The plasmid vector PV-GHGT35 used in the transformation contains two *cp4 epsps* gene expression cassettes within a single T-DNA that express the *cp4 epsps* coding sequence isolated from *Agrobacterium* sp. strain CP4. The *cp4 epsps* sequence encodes the CP4 EPSPS protein that consists of a single polypeptide of 455 amino acids with a calculated molecular weight of 47.6 kDa based upon the predicted amino acid sequence of the mature protein. The CP4 EPSPS protein produced in MON 88913 is targeted to the chloroplasts via an N-terminal fusion with the CTP2 to form a CTP2-CP4 EPSPS precursor protein. The precursor protein, produced in the cytoplasm, is then processed to remove the transit peptide upon translocation into the plant chloroplast, resulting in the mature protein (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Oblong and Lamppa, 1992).

A series of analyses were conducted to characterize the CP4 EPSPS protein isolated from cottonseed of MON 88913 and to establish the equivalence of the plant-produced protein to the *E. coli*-produced CP4 EPSPS protein standard that was used in studies to establish the safety of the protein. These analyses included N-terminal sequence analysis, immunoblotting and densitometry, and SDS-PAGE and densitometry. A brief summary of the results is provided below. The detailed results are presented in Appendix D, while information on the methods used is provided in Appendix B. The results of other analyses, including mass spectrometry, enzymatic activity, and glycosylation, were consistent with the results presented here.

The results of N-terminal sequence analysis of the plant-produced CP4 EPSPS protein were consistent with the expected sequence. The immunoblot analysis provided further data to confirm the identity of the plant-produced protein. SDS-PAGE (molecular weight) and immunoblot analysis were performed to evaluate the equivalence of the plant-produced protein to the *E. coli*-produced reference standard protein. The plant-produced CP4 EPSPS protein isolated from MON 88913 was equivalent to the *E. coli*-produced CP4 EPSPS reference standard protein based on comparable electrophoretic mobility and immunoreactivity.

Collectively, these data characterize and confirm the identity of the protein isolated from MON 88913 and establish the equivalence of the plant-produced CP4 EPSPS protein to the *E.coli*-produced CP4 EPSPS protein used in numerous safety studies (Section VIII.A.1.). These data also establish the equivalence of the CP4 EPSPS protein produced in MON 88913 to the CP4 EPSPS protein produced in a variety of major agricultural biotechnology crops, including Roundup Ready cotton, which is grown annually on more than 7.8 million acres within the U.S.

VI.D. Similarity of CP4 EPSPS to EPSPSs derived from food sources with a long history of safe consumption

The mature CP4 EPSPS protein present in MON 88913 is homologous to EPSPSs consumed in a variety of food and feed sources. The *cp4 epsps* coding sequence has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids. As shown in Table VI-2, the CP4 EPSPS protein is homologous to EPSPSs naturally present in plants, including food crops (*e.g.*, soybean and corn), and fungal and microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* (Mountain, 1989), which have a history of safe human consumption (Padgette et al., 1996; Harrison et al., 1996). The similarity of the CP4 EPSPS protein to EPSPSs in a variety of foods supports extensive human consumption of the family of EPSPS proteins and the lack of health concerns. Further, the ubiquitous presence of homologous EPSPS enzymes in food crops and common microbes establishes that EPSPS proteins, and their enzyme activity, pose no hazards for human consumption.

Table VI-2. Comparison of the deduced amino acid sequence of native CP4 EPSPS to that of other EPSPSs.

	soybean	**corn**	**petunia**	***E. coli***	***B. subtilis***	***S. cerevisiae***
CP4 EPSPS						
% sequence identity	26	24	23	26	41	30
% sequence similarity	51	49	50	52	59	54

VII. Phenotypic Evaluation

This section provides an evaluation of the phenotypic and crop compositional components of MON 88913 compared to MON 88913(-). These data provide information supporting a determination that MON 88913 is no more likely to pose a plant pest risk than conventional cotton.

An evaluation of the phenotype of MON 88913 was conducted to assess the phenotypic equivalence to MON 88913(-). The phenotypic evaluation is based on laboratory and greenhouse experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of cotton. Comparisons of phenotypic parameters between MON 88913 and a negative segregant control, MON 88913(-), and also to conventional cotton were conducted to establish the phenotypic and seed compositional equivalence of MON 88913. In each of these assessments, MON 88913 was compared to the negative segregant control, MON 88913(-), which was derived from MON 88913 and thus possesses similar varietal background genetics to MON 88913. In evaluating the phenotypic characteristics of MON 88913, data were collected that address specific pest potential characteristics that are considered by USDA-APHIS. These phenotypic characteristics have been grouped into five general categories: 1) dormancy, germination and emergence; 2) vegetative growth; 3) reproductive growth; 4) seed retention on plant; and 5) plant interactions with disease, insect, and abiotic stressors.

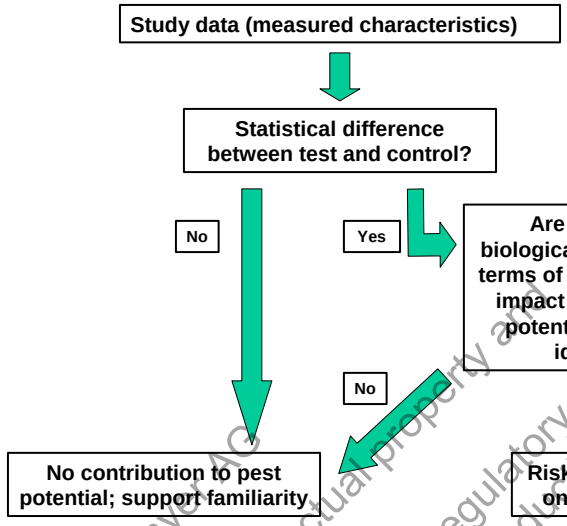
VII.A. Interpretation of Phenotypic and Ecological Interaction Data

Phenotypic, agronomic, and ecological data are useful to support the concept of phenotypic equivalence and familiarity as it relates to any ecological risk assessment. On the basis of these data, one can assess the probability of any increased pest potential of MON 88913, and whether the phenotype has been unintentionally changed beyond the intended introduced trait.

Measurement of phenotypic characteristics and environmental interactions provides data for a comparative assessment of ecological risk (pest potential) between a biotechnology-derived crop and an appropriate control. A tiered approach is used to assess whether a difference is, or is not, biologically meaningful. As such, evaluation of phenotypic characteristics is designed according to the biology of the crop using replicated plots at multiple locations with appropriate controls and commercial crop references. When no statistically significant differences in phenotypic characteristics are detected between the biotechnology-derived crop and an appropriate control, a conclusion of no contribution to pest potential can be made. If a statistically significant difference in a characteristic is detected, the magnitude of the difference would be considered (relative to the known ranges of values for the crop), and its effect on pest potential assessed to determine if it was biologically meaningful, as shown in Figure VII-1 below. All of the phenotypic data can be used to support crop familiarity (i.e., knowledge of and experience with the crop,

the trait, the receiving environment, and the interaction of these factors) (Hokanson et al., 1999), and a subset of the data, answering specific questions, can be used to evaluate any contribution to pest potential.

Figure VII-1. Schematic Diagram of Data Interpretation Methods:



***Consider direction and magnitude of change and interaction of differences, and whether the values fall within the range of known values for commercial cotton.**

Statistically significant changes in one characteristic are considered in terms of the direction of the change (i.e., contributing to or detracting from pest potential), its magnitude (outside the range of the control or reference organisms), and within the context of other observed changes. Interpretation of any detected differences in ecological risk assessment data should focus on those differences that are biologically meaningful (i.e., contribute to pest potential). Differences detected in a characteristic are considered alone and in the context of 1) whether or not trends were observed over locations; 2) differences were detected in other measured characteristics; 3) contributions to enhance any inherent pest potential of the crop; and 4) potential effects of trait transfer to a wild or weedy species.

For example, a significant difference in a growth characteristic may not be biologically meaningful in terms of weed potential if it is not outside the range typical for cotton or if a change in another parameter is in the direction toward lower weed potential. A careful assessment must be used to distinguish between meaningful changes toward increased pest potential and differences associated with natural plant variation or random experimental error. A finding of no meaningful difference can be concluded only after a thorough evaluation of all the data collected on the characteristics measured.

VII.B. Agronomic Characteristics

As a significant part of the phenotypic evaluation of MON 88913, key agronomic characteristics were evaluated, including plant growth and development under field conditions, crop productivity, harvest quality and seed germination.

VII.B.1. Field Phenotypic Analysis

Plant growth and development characteristics were assessed during growth in the field, and harvest materials were assayed in the laboratory to identify any unintended phenotypic effects or ecological interactions in MON 88913 relative to MON 88913(-) and conventional cotton. The purpose of this field evaluation was to assess whether the presence of the *cp4 epsps* coding sequence or the presence of the CP4 EPSPS protein altered the phenotypic characteristics or the plant-insect, plant-pathogen, or plant-abiotic stressor interactions of MON 88913 compared to MON 88913(-). Certain growth, reproduction, and pre-harvest seed loss characteristics (such as boll drop) can be used for an assessment of enhanced weed potential of MON 88913. Detected differences were evaluated alone, in consideration of other observed differences, and for trends across locations as described above in Section VII.A.

Field trials were conducted in 2002 at 14 locations across the U.S. cottonbelt to thoroughly evaluate phenotypic characteristics (Tables VII-1, VII-2). These fourteen locations provided a diverse range of environmental and agronomic conditions representative of the U.S. cottonbelt encompassing the majority of commercial cotton production, including regions where MON 88913 would be anticipated to be produced. The methods and detailed results of these comparisons are presented in Appendices A and C, respectively, and are summarized below.

A randomized complete block design with four replications was employed for the comparisons and analysis. A total of 41 different phenotypic characteristics were evaluated including 11 characteristics during plant growth and development, 20 characteristics from plant mapping, four characteristics from boll/seed measurements, and six boll and fiber quality characteristics (Table VII-2). In addition, observational data on the presence and any differential response to biotic (pests and disease) and abiotic stressors were collected. These measurements are well known to cotton researchers and can provide supplementary data to assess plant pest potential.

Out of a total of 458 comparisons between MON 88913 and MON 88913(-) by field location, 19 differences were detected at $p \leq 0.05$. There were no differences detected at any location between MON 88913 and MON 88913(-) for six of 11 plant growth and development characteristics measured, 13 of 20 plant map characteristics, two of four boll/seed measurements and three of six boll and fiber quality characteristics (Appendix C). Most observed differences occurred for a single characteristic at a single field location. Furthermore, it is important to note that a frequency of differences of 4.15% (19/458 x 100) was less than the 5% level of error standard set for statistical significance,

and further suggests that the transformation produced no significant impacts on the measured growth and development characteristics.

When all data were pooled across locations, a single difference in the growth and development characteristics was observed. The date until 50% flowering was later for MON 88913 compared to MON 88913(-) (64 vs. 63 days after planting, respectively) (Table VII-3). This difference was one day at most sites, has little biological meaning in terms of plant weed potential, and could be because of small differences in the background genetics between MON 88913 and MON 88913(-).

When data were pooled across sites, no differences between MON 88913 and MON 88913(-) were detected for any of the measured plant map characteristics (Table VII-4). A single difference was observed across sites in the boll/seed measurements. The seed index of MON 88913 was lower than MON 88913(-) (9.56 vs. 9.83g per 100 fuzzy seed, respectively) (Table VII-5). This difference was approximately 0.3 g per 100 fuzzy seed, likely has little biological meaning in terms of plant weed potential, and the values fall within the range of commercial cotton varieties (Silvertooth et al., 1999). When boll and fiber quality data were analyzed across all sites, MON 88913 boll size was smaller and micronaire was less compared to MON 88913(-) (4.56 vs. 4.70 g per boll and 3.758 vs. 3.881 mike units, respectively) (Table VII-6). Small changes in seed size and micronaire are unlikely to increase weed potential, and both micronaire values are agronomically equivalent, falling within the premium target range of 3.7 – 4.2 (USDA, 2001).

Each field site also was rated at four times during the season for specific insect pests, diseases and abiotic stressors, although not all sites were rated for each pest or stressor because a given pest or stressor may not have been present. The purpose of these evaluations was to assess whether plant-pathogen or plant-pest interactions of MON 88913 were altered compared to MON 88913(-) such that the pest potential was altered. Fourteen insect categories (species or group), four disease categories and ten abiotic stressors were evaluated. Out of 106 insect observations, only one site reported a difference in susceptibility between MON 88913 and MON 88913(-). Beet armyworm was a severe stressor at the Tift County, GA location on the first observation date and the cooperator noted that in one of the four replications the MON 88913 plot had more damage than the MON 88913(-) plot. This was not observed in other replications, or at the other observations times or locations, suggesting that this may have been due to the location of the field plot or a localized infestation, rather than a function of the plants. Out of seven disease and 38 abiotic stressor observations, no differences were detected between MON 88913 and MON 88913(-) (Appendix C, Table C-10). These results support the conclusion that environmental interactions of MON 88913 are not expected to be different than that of other cotton.

The phenotypic data from these field and laboratory assessments indicate that, compared to MON 88913(-), MON 88913 does not possess characteristics that would confer a selective advantage that would result in increased weed or pest potential. The data on environmental interactions also indicate that the Roundup Ready Flex cotton MON 88913 does not confer any increase in pest potential to cotton, nor suggest any

changes in the interactions between MON 88913 and the field environment. These conclusions were additionally supported by the lack of detectable trends for differences in susceptibility or tolerance to specific insect, disease, or abiotic stressors across locations. These data suggest no difference in pest potential between MON 88913 and MON 88913(-) as no consistent trends across sites were observed for differences in susceptibility or tolerance to specific disease, insect, or abiotic stressors (Appendix C, Table C-10). Taken together as a whole, the data also support familiarity and phenotypic equivalence of MON 88913 to MON 88913(-).

Table VII-1. Field Phenotypic Evaluation Sites for MON 88913 During 2002.

Location	Location Code	USDA-APHIS Notification Number
Rapides Co., Louisiana	AL	02-016-27n
Limestone Co., Alabama	BM	02-022-54n
Florence Co., South Carolina	FL	02-025-01n
Mississippi Co., Arkansas	KS	02-028-28n
Lubbock Co., Texas	LB	02-025-08n
Washington Co., Mississippi	LL	02-025-02n
Pinal Co., Arizona	MR	02-018-16n
Fort Bend Co., Texas	NV	02-025-08n
San Patricio Co., Texas	PL	02-004-11n
Pemiscot Co., Missouri	PV	02-022-55n
Edgecombe Co., North Carolina	RL	02-025-01n
Oktibbeha Co. Mississippi	SV	02-025-02n
Tift Co., Georgia	TF	02-025-07n
Obion Co., Tennessee	UC	02-023-15n

Table VII-2. Phenotypic Characteristics Evaluated in U.S. Field Trials During 2002.

Category and Characteristic	Evaluation Timing	Evaluation Description
Plant growth and development		
1st and 2nd emergence counts	Approx. 7 and 14 days after planting	Number of plants emerged (≥2 cotyledon stage) per 30 ft of row
1st, 2nd and 3rd plant height	Approx. 4, 8, and 12 weeks after planting	Distance from soil to uppermost terminal meristem
1st, 2nd, and 3rd plant vigor	Approx. 4, 8, and 12 weeks after planting	Rated on a scale of 1-10, where 1 = poor and 10 = very good vigor
Days to 50% flowering	Flowering	Days from planting when white flowers occurred on 50% of the plants
Node-above-cracked-boll	In-season	Days from planting when 50% of the sampled plants reached node-above-cracked boll = 7
Yield	Harvest	Cottonseed yield
Plant mapping		
Height	Harvest	Distance from base (soil level) of the plant to uppermost terminal meristem
Nodes	Harvest	Number of mainstem nodes on the plant
Height per node	Harvest	Calculated as height / node
Total bolls	Harvest	Number of bolls on 10 sampled plants
Position 1 bolls (total, normal and abnormal)	Harvest	Number of total, normal and abnormal position 1 bolls on 10 sampled plants
Position 2 bolls (total, normal and abnormal)	Harvest	Number of total, normal and abnormal position 2 bolls on 10 sampled plants
Vegetative bolls	Harvest	Number of bolls per plant
Percent abnormal bolls	Harvest	Percent of total bolls that are abnormal

Table VII-2 (Continued). Phenotypic Characteristics Evaluated in U.S. Field Trials During 2002.

Category and Characteristic	Evaluation Timing	Evaluation Description
Plant mapping		
Position 1 & 2 bolls (%) for nodes 4–9, 10-14, 15-19, and 20-26	Harvest	Percent of total bolls at nodes 4–9, 10-14, 15-19, and 20-26 at position 1 or position 2 from 10 plants sampled
Boll and seed counts		
Seed index (g/100 seed)	Post-harvest	Mass of 100 ginned seed
Total seed per boll	Post-harvest	Number of seeds in a boll calculated from 25 boll sample
Mature seed per boll	Post-harvest	Number of mature seeds in a boll calculated from 25 bolls sample
Immature seed per boll	Post-harvest	Number of immature seeds in a boll calculated from 25 boll sample
Fiber quality		
Boll size (g/boll)	Post-harvest	Mass of a single boll calculated from 25 boll sample
Micronaire (mike units)	Post-harvest	Measure of fiber surface area related to fiber perimeter, maturity and surface characteristics. Measured as air pressure through a 3.2g sample of lint.
Elongation (%)	Post-harvest	Measure of the stretch of 1/8" gauge of fiber. A stelometer applies tension to both ends of the fiber and the breaking point is the elongation.
Strength (g/tex)	Post-harvest	Strength of a bundle of fibers. One tex is the mass in grams of 1,000 meter of fiber.

Table VII-2 (Continued). Phenotypic Characteristics Evaluated in U.S. Field Trials During 2002.

Category and Characteristic	Evaluation Timing	Evaluation Description
Fiber Quality		
Span length 2.5 and 50 (inches)	Post-harvest	An optical measurement of fiber length using a digital fibrograph. From a beard of fibers, the mass at various cross sections along the length of the beard is determined. 50% span length is the length of fiber where the cross-sectional amount is reduced to 50%. This measurement is in inches.
Insect and disease observations		
1st, 2nd, 3rd, and 4th Observations	Approximately 4, 8, 12, and 16 weeks after planting	Qualitative assessment of insect and disease incidence in the plots.

Table VII-3. Plant Growth and Development Data Across 14 Locations During 2002.

Characteristic	Unit	MON 88913(-)	MON 88913
1st Emergence Count	# per 30 ft	104	101
2nd Emergence Count	# per 30 ft	130	132
1st Height Measurement	cm	19	20
1st Vigor Measurement[1]	Rating	8	8
2nd Height Measurement	cm	63	63
2nd Vigor Measurement[1]	Rating	9	9
3rd Height Measurement	cm	89	90
3rd Vigor Measurement[1]	Rating	9	9
Days Until 50% Flowering	DAP	63	64*+
Node Above Cracked Boll	DAP	118	118
Seedcotton Yield	lbs/acre	2135	2226

*Indicates a statistical difference was detected *($p \leq 0.05$)* between MON 88913 and MON 88913(-) for a given characteristic across locations.

+Indicates a significant location by characteristic interaction (see Appendix C for explanation). Linear contrast was used to compare MON 88913 to MON 88913(-). Material and a time-by-location interaction was detected for this characteristic.

[1]Rated on a scale of 1-10, where 1 = poor and 10 = very good vigor .

Table VII-4. Plant Map Data Across 14 Locations During 2002.

Characteristic	Unit	MON 88913(-)	MON 88913
Height at Plant Mapping	cm	88	88
Nodes	#	20	20
Height per Node	cm / node	4.6	4.6
Total Bolls	# per 10 plants	74	72
Total Position 1 Bolls	# per 10 plants	50	50
Total Normal Position 1 Bolls	# per 10 plants	48	48
Total Abnormal Position 1 Bolls	# per 10 plants	2	2
Total Position 2 Bolls	# per 10 plants	24	22
Total Normal Position 2 Bolls	# per 10 plants	23	21
Total Abnormal Position 2 Bolls	# per 10 plants	1	1
Vegetative Bolls	# per plant	1	1
Abnormal Bolls	%	4	5
Position 1 Bolls on Nodes 4 to 9	%	52	53
Position 1 Bolls on Nodes 10 to 14	%	44	48
Position 1 Bolls on Nodes 15 to 19	%	31	28
Position 1 Bolls on Nodes 20 to 26	%	17	20
Position 2 Bolls on Nodes 4 to 9	%	33	31
Position 2 Bolls on Nodes 10 to 14	%	30	30
Position 2 Bolls on Nodes 15 to 19	%	19	26
Position 2 Bolls on Nodes 20 to 26	%	13	12

No statistical differences were detected *($p \leq 0.05$)* between MON 88913 and MON 88913(-) for a given characteristic across locations.

Table VII-5. Boll/Seed Measurements Across 14 Locations During 2002.

Characteristic	Unit	MON 88913	MON 88913(-)
Seed Index	g per 100 fuzzy seed	9.56*+	9.83
Total Seed per Boll	# per boll	31	29
Mature Seed per Boll	# per boll	28	27
Immature Seed per Boll	# per boll	2	2

*Indicates a statistical difference was detected *($p \leq 0.05$)* between the MON 88913 and MON 88913(-) for a given characteristic across locations.

+Indicates a significant location by characteristic interaction (see Appendix C for explanation). Linear contrast was used to compare the test to the control. Material and a seed index-by-location interaction was detected for this characteristic.

Table VII-6. Boll and Fiber Quality Characteristics Across 14 Locations During 2002.

Characteristic	Unit	MON 88913	MON 88913(-)
Boll size	g	4.56*	4.70
Micronaire	mike units[1]	3.758*	3.881
Elongation	%	6.801	6.777
Strength	g/tex[1]	19.701	19.781
Span length 2.5%	inches	1.128	1.129
Span length 50%	inches	0.553	0.555

*Indicates a statistical difference was detected *($p \leq 0.05$)* between MON 88913 and MON 88913(-) for a given characteristic across locations.

[1]See Table VII-2 for explanation.

VII.B.2. Seed Dormancy Characteristics

Seed dormancy is an important characteristic that is often associated with plants that are weeds (Anderson, 1996). Dormancy mechanisms, including hard seed, vary with species and tend to involve complex processes. Standardized germination assays of the Association of Official Seed Analysts (AOSA, 1998) are used as a baseline to measure the germination potential of cottonseed. Changes were not expected in the dormancy and germination characteristics of MON 88913 compared to MON 88913(-) or to conventional cotton based on the ubiquitous nature of EPSPS proteins in plants, the mechanism of the EPSPS enzyme, and the commercial history of Roundup Ready cotton.

Seed dormancy characteristics were compared between MON 89913 and MON 88913(-) to assess the potential impact of the presence of the DNA insert or the CP4 EPSPS protein produced in MON 88913 on cottonseed dormancy. In this evaluation, all tests were conducted as replicated comparisons between MON 88913 and MON 88913(-). Six conventional cotton varieties were included to provide baseline values common to commercial conventional cotton. The seed dormancy characteristics evaluated included germination under the optimal 20/30ºC temperature regime following standard guidelines (AOSA, 1998), plus six other temperature regimes of 10, 20, 30, 40 10/20, and 10/30°C (Table VII-7). In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours. The temperature inside each growth chamber was monitored and recorded every 15 minutes using Watchdog™ 110 Data Loggers (with an accuracy of +/- 0.7°C). The tested seed were produced during 2002 at three field locations within the U.S. cottonbelt: Baldwin County, AL; Tulare County, CA; and Clarke County, GA, representing environmentally relevant conditions for cotton production. The experimental methods and detailed results of these comparisons are presented in Appendices A and C, respectively, and are summarized below.

Table VII-7. Seed Germination Parameters Evaluated.

Category and Characteristic	Evaluation Regime	Evaluation Description
Seed Germination / Dormancy Observations	**Temperature °C†**	
Normal Germinated	20/30, 10, 10/20, 20, 30, 40, 10/30	Exhibiting normal germination development characteristics.
Abnormal Germinated	20/30, 10, 10/20, 20, 30, 40, 10/30	Germinated, but lacking shoot or root, or diseased.
Germinated	10, 10/20, 20, 30, 40, 10/30	Possesses radicle ≥ 2mm beyond seed coat.
Dead	20/30, 10, 10/20, 20, 30, 40, 10/30	Visibly deteriorated, soft.
Viable Hard	20/30, 10, 10/20, 20, 30, 40, 10/30	Non-imbibed, hard.
Viable Firm Swollen	20/30, 10, 10/20, 20, 30, 40, 10/30	Imbibed, firm and swollen.

†Constant temperature maintained at ~10, 20, 30, or 40°C, or alternating temperatures of 10/20, 10/30, or 20/30°C. In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours.

Across most temperature regimes, the percent germination rates for cottonseed from the AL and GA locations were approximately half those of cottonseed from the CA location. MON 88913, MON 88913(-) and conventional cottonseed were all affected similarly. These results were anticipated because cotton is primarily grown in Alabama and Georgia for lint production, and not necessarily for commercial planting seed. Humid conditions, typical of Alabama and Georgia, can lead to degradation of seed quality. Although seed quality at AL and GA was poor by seed production standards, it is representative of areas within the cottonbelt where MON 88913 is anticipated to be produced primarily for lint. The difference in germination was not found to be correlated with the Roundup Ready trait as MON 88913, MON 88913(-) and the conventional reference cottonseed were all affected (Appendix C, Tables C-2, 3, 4).

Out of 87 comparisons between MON 88913 and MON 88913(-), 75 were not statistically significant at $p \leq 0.05$. No differences between MON 88913 and MON 88913(-) were detected for seed dormancy-related characteristics, such as hard seed, with seed from any location. Of the 12 significant differences detected, 10 occurred in seed from the AL and GA locations and two in seed from the CA location (Appendix C, Tables C-2, 3, 4). Specifically, one significant difference was observed under the optimal temperature regime (20/30ºC); cottonseed from the AL location showed reduced germination in MON 88913 relative to MON 88913(-). The difference was not detected in cottonseed from the GA or CA locations. Four additional differences were detected

with seed from the AL and GA locations where percent germination in MON 88913 was reduced compared to MON 88913(-). In each of these five specific cases, there was an accompanying rise in the number of dead seed accounting for five additional differences. Decreased germination accompanied by more dead seed with no changes in hard or viable firm swollen seed would not indicate increased weed potential of MON 88913. More importantly, with the exception of the AL location at 40°C, where a difference was detected, percent germination for MON 88913 was within the range of values generated for the commercial conventional cottonseed produced in the same field trial as MON 88913.

The remaining two statistical differences were detected between MON 88913 and MON 88913(-) in the 10/20°C temperature regime for percent dead seed (CA location) and percent viable firm swollen seed (GA location). These differences were very small and values from MON 88913 were within the ranges observed from conventional cotton produced at each respective location. In particular, there was a lack of differences in hard seed from any location which could correspond to dormancy (Appendix C, Tables C-2, 3, 4). Therefore, these differences are unlikely to be biologically meaningful.

The lack of meaningful differences between MON 88913, MON 88913(-), and conventional cotton varieties indicate that the presence of the DNA insert or the presence of the CP4 EPSPS protein did not alter the seed dormancy and germination characteristics of MON 88913. These data suggest that there was no change in the weed potential of MON 88913 as a result of increased dormancy or from changes in germination characteristics, further supporting phenotypic equivalence and familiarity.

VII.B.3. Confirmatory Field Observations

The agronomic evaluation of MON 88913 also included observational information on disease/pest susceptibility and phenotypic assessments from other product evaluation field trials conducted over several growing seasons (see Appendix A for a listing of USDA field trial final reports). These observations provide confirmatory information to the quantitative agronomic characterization data provided in this section (Section VII.). Field trials were conducted with MON 88913 during the years 2002-2003 under various product development protocols. These trials were established for the purpose of testing agronomic performance, crop efficacy and glyphosate tolerance, genetic background combining ability, developing weed control programs and assessing volunteer cotton incidence, assessing glyphosate residue levels, production of materials for product characterization studies, etc. The field designs and protocols for these trials varied according to purpose, with some trials replicated and others nonreplicated, most often comparing MON 88913 to MON 88913(-). Results of some of these trials have been presented at the Cotton Beltwide meetings (Subramani et al., 2002; May et al., 2003; Keeling et al., 2003; Martens et al., 2002; 2003; Croon et al., 2003).

In addition, in order to generate materials for *in planta* CP4 EPSPS protein characterization and quantification, molecular characterization, cottonseed composition,

and evaluation of cottonseed dormancy, replicated field trials were conducted at four locations in the U.S. during 2002 (Appendix C, Section C.3.). The plants in these trials were grown under agronomic and cultural practices that are typical of cotton production within these regions. The field plots were periodically monitored (approximately every four weeks after planting) and observed for plant stressors, including susceptibility to common insect pests and pathogens. Insect pests were present at all sites and slight to moderate infestations were observed. Pesticides were applied in response to the insect pests according to normal agricultural practices for the location. Disease problems were not observed at any of the sites and there were no meaningful observed differences among the test, control and reference cotton plots with respect to arthropod damage.

VII.B.4. Reproductive Tolerance and Floral Phenotypic Characteristics

The assessment of phenotypic characteristics of MON 88913 established, with the exception of glyphosate tolerance, that there were no observed differences between MON 88913 and cotton of similar background genetics that does not contain the DNA insert. MON 88913 was designed to enhance the reproductive tolerance to glyphosate compared to that currently observed in Roundup Ready cotton. Therefore, a confirmatory investigation was sponsored by Monsanto to compare pollen and floral morphology in glyphosate-treated and untreated MON 88913, MON 88913(-) and Roundup Ready cotton. Data were generated to determine the effects of over-the-top, sequential applications of glyphosate on MON 88913 on pollen viability, pollen availability, and floral morphology (Table VII-8). The details of the methods and the full results are presented Appendices B and C, respectively. A summary of the methods and results comparing untreated MON 88913 to MON 88913(-), and treated MON 88913 to Roundup Ready cotton are presented below.

MON 88913, MON 88913(-), and Roundup Ready cotton, were grown in a greenhouse. One set of MON 88913, MON 88913(-) and Roundup Ready cotton plants remained untreated while Roundup WeatherMAX® was sequentially applied over the top of a set of MON 88913 and Roundup Ready cotton plants at three different growth stages at 1.5 lb. ae/A per application (Appendix B). Plants were sprayed at approximately the four-leaf (node) stage, 8-leaf (node) stage, and 12-leaf (node) stage. Anther dehiscence, anther height, stamen length (anther + filament), staminal column height, pollen grains on stigmatic lobe, pollen deposition, and pollen viability (two methods) were evaluated.

No difference was detected in anther dehiscence, stamen length, staminal column height, the number of pollen grains attached to a stigmatic lobe, pollen deposition rating or percent pollen viability (either staining method) between untreated MON 88913 and untreated MON 88913(-) (Table VII-9). Untreated MON 88913 anther height as a percent of pistil length was greater than untreated MON 88913(-) (Table VII-9). This small percentage difference (4%) would convert to a relatively minor actual height difference and would have little biological meaning in terms of flower morphology or

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function; this was corroborated by the field plant mapping data in the previous field phenotypic analyses.

MON 88913 demonstrated significantly increased reproductive tolerance and pollen viability compared to Roundup Ready cotton under these herbicide treatments. Percent pollen viability was significantly greater in treated MON 88913 compared to treated Roundup Ready cotton. Furthermore, the number of pollen grains attached to the stigmatic lobe was markedly increased in treated MON 88913 over treated Roundup Ready cotton (Appendix C).

Table VII-8. Reproductive Phenotypic Characteristics Evaluated.

Reproductive Morphology/ Floral Ontogeny	**Measurements following sequential glyphosate applications**	**Description of measurements**
Anther dehiscence	Rating scale	Rating based on percent dehiscence.
Anther height	Anther height as percent of pistil length	Measurement in centimeters.
Stamen length	Anther + filament	Measurement in centimeters.
Staminal column height	Staminal column + ovary	Measurement in centimeters.
Reproductive Morphology/ Pollen deposition		
Pollen grains on stigmatic lobe	Pollen count	Number of pollen grains on stigmatic lobe.
Pollen deposition rating	Rating based on location of pollen distribution on stigmatic lobe	Rating scale: 0-3.
Pollen viability	Brewbacker & Kwack[1] staining method	Counts of germinated pollen on medium
Pollen viability	Alexander[2] stain dye assay for viability	Counts of aborted and non-aborted pollen

[1]Brewbaker and Kwack, 1963.
[2]Alexander, 1969, 1980.

Table VII-9. Floral Phenotypic Characteristics.

Characteristic	Measurement	MON 88913	MON 88913(-)
Anther dehiscence[1]	Rating Scale	3.7	3.5
Anther height[2]	% of pistil length	100*	96
Stamen length[3]	Millimeters	5.9	5.9
Staminal column height[4]	Millimeters	11.6	11.9
Pollen grains on stigmatic lobe	Number	139	142
Pollen deposition[5]	Rating	2.6	2.6
Pollen viability[6]	Mean % viable pollen	85	90
Pollen viability[7]	Mean % viable pollen	95	95

*Indicates a difference was detected ($p \leq 0.05$) between the MON 88913 and MON 88913(-) for a given characteristic. The statistical analysis was conducted to compare all treatments included in the experiment (Appendix C). For this table, the statistical differences between untreated MON 88913 and untreated MON 88913(-) were extracted from the complete analysis.

[1]Mean anther dehiscence ratings where 0 = 0% dehisced; 1 = 25% dehisced; 2 = 50% dehisced; 3 = 75% dehisced; 4 = 100% dehisced (open).

[2]Mean of the uppermost anther height as a percent of pistil length; anther height as % of pistil length calculated from the raw data = 100 – ({[pistil length – uppermost anther height] / pistil length} x 100).

[3]Mean stamen length (Anther + filament)

[4]Mean staminal column length (including ovary).

[5]Mean pollen deposition rating where 0 = no pollen attached; 1 = distribution over lower 2/3 of stigma; 2 = distribution over upper 2/3 of stigma; 3 = even distribution over entire stigma.

[6]Brewbaker and Kwack staining method.

[7]Alexander staining method.

VII.B.5. Conclusions of the Phenotypic Comparisons

Data developed from these investigations were collected from a broad range of environmental conditions and agronomic practices that MON 88913 would likely encounter. These data include observations that are typically recorded by plant breeders and agronomists to evaluate the qualities of cotton. The characteristics measured provide crop biology data useful in establishing a basis to assess equivalence and familiarity in the context of ecological risk assessment. The phenotypic characteristic data detected no biologically meaningful differences between MON 88913, MON 88913(-) and conventional cotton and support a conclusion of phenotypic equivalence as it relates to familiarity, and a lack of increased weed potential. Detected differences were considered alone, in consideration of other observed differences, and for trends across locations. Each detected difference was considered with respect to its impact for increasing any inherent weed potential of the crop and if the trait were to be transferred to a wild relative. The phenotypic data indicate that MON 88913 possesses no fitness advantage comparable to other cotton that would result in increased weed potential.

VII.C. Composition of Cottonseed of MON 88913

Compositional analysis is useful indicate whether levels of nutrients, antinutrients, toxicants or other components of MON 88913 are altered relative to the appropriate control and to commercial conventional cotton.

In order to assess whether there was any effect on the composition of the cottonseed, a compositional analysis was conducted of delinted cottonseed collected from MON 88913 grown under replicated field conditions in the U.S. at four sites (Appendix C, Section C.3.). MON 88913 was compared to MON 88913(-), which has background genetics representative of the test material but does not contain the DNA insert or produce the CP4 EPSPS protein. Sixteen commercial conventional cotton varieties produced in the same field trial alongside MON 88913 and MON 88913(-) were also analyzed as references to produce a 99% tolerance interval for conventional cotton. The field experimental design and compositional methods are described in Appendices B and C, and are summarized below.

Analyses were conducted on the cottonseed to measure proximates (protein, total fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, total dietary fiber (TDF), amino acids, fatty acids (C8-C22), cyclopropenoid fatty acids (malvalic acid, sterculic acid, and dihydrosterculic acid), vitamin E, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), gossypol (free and total), and aflatoxins (B1, B2, G1, and G2). In addition, carbohydrates and calories were determined by calculation.

In all, 69 different components were evaluated as part of the nutritional assessment of cottonseed derived from MON 88913. Of the 69 components evaluated, 50% of the

observations for 16 of the components were below the assay detection limit and were therefore excluded from the statistical analysis. As a result, 53 components were statistically analyzed. A total of 265 comparisons were made: 53 comparisons for each of the five statistical analyses (four sites individually plus all sites combined). MON 88913 was compared to MON 88913(-) to determine statistically significant differences at a significance level of $p \leq 0.05$. In addition, for those comparisons in which MON 88913 was statistically different from MON 88913(-), the range of values for MON 88913 was compared to the 99% tolerance interval (with 95% confidence) of the commercial conventional reference varieties to determine if the test values fell within the population of commercial cotton.

There were no statistically significant differences between MON 88913 and MON 88913(-) for 236 of the 265 comparisons, including fifteen of eighteen amino acids, six of the ten fatty acids statistically analyzed, dihydrosterculic acid, iron, magnesium, phosphorus, ash, protein, calories, carbohydrates, vitamin E, acid detergent fiber, neutral detergent fiber, total dietary fiber, free gossypol, and total gossypol (Appendix E). Of the 29 comparisons found to be statistically different, 5%, or approximately 13 (0.05 x 265), were expected based on chance alone. Statistically significant differences ($p \leq 0.05$) between MON 88913, and MON 88913(-) were observed in one of the five comparisons for tryptophan, glycine, 16:0 palmitic acid, 18:0 stearic acid, malvalic acid, sterculic acid, crude fiber, moisture, copper, and zinc; in two of the five comparisons for phenylalanine, calcium, manganese, and fat; in three of the five comparisons for sodium and 18:2 linoleic acid; and in all five comparisons for 18:1 oleic acid (Table VII-10).

These last two fatty acid components, 18:2 linoleic acid and 18:1 oleic acid, showed a compositional difference between MON 88913 and MON 88913(-) in cottonseed produced at greater than half of the sites, and were statistically different in the combined site analysis. However, the differences between MON 88913 and MON 88913(-) for the components were small, (3.8 to 5.0%) and (8.5 to 13.7%), respectively. These differences could be explained by differences in the background genetics between MON 88913 and MON 88913(-). Importantly, the oleic and linoleic acid content of cottonseed of MON 88913 is not outside the range of expected values for these components in cotton (Table VII-10). These and the other observed differences are unlikely to be biologically meaningful because the range of values for all components associated with the statistically significant differences were found to fall within the 99% tolerance interval for the commercial varieties planted in the same field trials as MON 88913 and MON 88913(-), with the exception of moisture in the combined site comparison. The range of test values for moisture did, however, fall within published ranges for commercial cottonseed (Table VII-10).

These results demonstrate that the levels of key nutrients and other components of cottonseed of MON 88913 are within the expected range for conventional cotton. In addition, the background genetics of MON 88913 and MON 88913(-) cottonseed are expected to be genetically similar but not 100% identical, further providing a practical context for minor differences noted between MON 88913 and MON 88913(-). In this context, minor differences within the range of expected values for commercial cotton

were unlikely to be biologically meaningful, and the compositional components of cottonseed from MON 88913 were considered to be compositionally equivalent to cottonseed of conventional cotton. The statistical evaluation of the compositional data is summarized in Appendix E. Table VII-10 below presents a summary of the values that were statistically significant.

Table VII-10. Summary of Statistical Differences *(p≤0.05)* for the Comparison of MON 88913 to MON 88913(-), Plus Commercial Varieties.

Site / Component[a]	Mean [b] MON 88913	Mean [b] MON 88913(-)	Mean Difference [% of MON 88913(-)] [b]	Significance (*p-value*) [b]	MON 88913 Range [b]	Commercial 99% T. I. [Lower, Upper] [b]	Literature Range [d]
Alabama							
18:1 Oleic (% Total FA)	16.60	18.89	-12.1	0.002	16.35 – 17.01	[10.59,21.29]	15.17 [1]–19.94 [1]
18:2 Linoleic (% Total FA)	53.88	51.69	4.2	<0.001	53.54 – 54.32	[48.89,61.11]	49.07 [1]–59.1 [2]
Manganese (mg/kg dwt)	19.31	17.77	8.7	0.031	18.45 – 19.98	[4.69,26.45]	10 [3]–20.1 [4]
Sodium (% dwt)	0.10	0.080	30.5	0.017	0.087 – 0.12	[0,0.17]	0.03 [4]–0.31[3]
Zinc (mg/kg dwt)	43.39	40.96	5.9	0.032	41.22 – 44.70	[17.12,58.50]	28.9 [4]–37 [5]
Total Fat (% dwt)	21.33	22.41	-4.8	0.004	21.04 – 21.91	[15.16,28.44]	16.9 [4]–26.8 [1]
California							
16:0 Palmitic (% Total FA)	24.33	23.70	2.6	0.031	23.56 – 24.69	[20.76,26.19]	18.4 [2]–26.18 [1]
18:1 Oleic (% Total FA)	19.77	21.61	-8.5	0.026	19.05 – 20.35	[10.59,21.29]	15.17 [1]–19.94 [1]
Malvalic (% Total FA)	0.26	0.34	-25.3	0.003	0.24 – 0.27	[0.16,0.58]	0.7 [6]–1.5 [6]
Sterculic (% Total FA)	0.26	0.28	-7.9	0.020	0.25 – 0.28	[0.18,0.40]	0.3 [6]–0.5 [6]
Crude Fiber (% dwt)	16.06	16.87	-4.8	0.020	14.96 – 16.79	[13.34,24.17]	20.8 [3]

Table VII-10 (Continued). Summary of Statistical Differences *($p \leq 0.05$)* for the Comparison of MON 88913 to MON 88913(-), Plus Commercial Varieties

Site / Component [a]	Mean [b] MON 88913	Mean [b] MON 88913(-)	Mean Difference [% of MON 88913(-)] [b]	Significance (*p-value*) [b]	MON 88913 Range [b]	Commercial 99% T. I. [Lower , Upper] [b]	Literature Range [d]
California							
Calcium (% dwt)	0.16	0.18	-9.2	0.008	0.16 – 0.17	[0.074,0.22]	0.1 [4]–0.17 [5]
Sodium (% dwt)	0.060	0.037	61.5	0.002	0.053 – 0.068	[0,0.17]	0.03 [4]–0.31 [3]
Georgia							
Glycine (% Total AA)	4.34	4.37	-0.6	0.040	4.33 – 4.38	[4.21,4.64]	3.7 [7]–4.6 [7]
18:0 Stearic (% Total FA)	2.73	2.64	3.5	0.045	2.68 – 2.77	[2.18,3.17]	2.2 [2]–2.88 [1]
18:1 Oleic (% Total FA)	18.57	20.67	-10.2	0.015	17.93 – 19.14	[10.59,21.29]	15.17 [1]–19.94 [1]
Calcium (% dwt)	0.14	0.12	11.2	0.014	0.13 – 0.15	[0.074,0.22]	0.1 [4]–0.17 [5]
Sodium (% dwt)	0.045	0.10	-56.2	0.001	0.037 – 0.051	[0,0.17]	0.03 [4]–0.31 [3]
Texas							
Phenylalanine (% Total AA)	5.65	5.59	1.0	0.023	5.61 – 5.67	[5.43,5.82]	5.0 [7]–6.2 [7]
18:1 Oleic (% Total FA)	19.51	22.59	-13.7	0.012	18.71 – 20.72	[10.59,21.29]	15.17 [1]–19.94 [1]
18:2 Linoleic (% Total FA)	51.63	49.17	5.0	0.005	50.80 – 52.61	[48.89,61.11]	49.07 [1]–59.1 [2]
Copper (mg/kg dwt)	6.78	6.28	7.9	0.036	6.59 – 6.96	[2.01,12.94]	9.9 [4]–54 [3]
Total Fat (% dwt)	22.96	21.84	5.1	0.024	22.52 – 23.54	[15.16,28.44]	16.9 [4]–26.8 [1]

Table VII-10 (Continued). Summary of Statistical Differences *($p \leq 0.05$)* for the Comparison of MON 88913 to MON 88913(-), Plus Commercial Varieties

Site / Component[a]	Mean [b] MON 88913	Mean [b] MON 88913(-)	Mean Difference [% of MON 88913(-)] [b]	Significance (*p-value*) [b]	MON 88913 Range [b]	Commercial 99% T. I. [Lower , Upper] [b]	Literature Range [d]
Combined Site							
Phenylalanine (% Total AA)	5.64	5.60	0.8	0.031	5.53 – 5.75	[5.43,5.82]	5.0 [7]–6.2 [7]
Tryptophan (% Total AA)	1.10	1.14	-3.4	0.029	1.03 – 1.23	[0.94,1.26]	1.0 [7]–1.4 [7]
18:1 Oleic (% Total FA)	18.61	20.94	-11.1	0.003	16.35 – 20.72	[10.59,21.29]	15.17 [1]–19.94 [1]
18:2 Linoleic (% Total FA)	52.36	50.42	3.8	<0.001	49.66 – 54.32	[48.89,61.11]	49.07 [1]–59.1 [2]
Manganese (mg/kg dwt)	15.34	14.64	4.8	0.024	12.37 – 19.98	[4.69,26.45]	10 [3]–20.1 [4]
Moisture (% fwt)	6.39	6.22	2.7	0.013	5.65 – 7.34	[4.51,7.21]	5.4 [1]–10.1 [1]

[a] dwt=dry weight; AA=amino acids; FA=fatty acids; fwt=fresh weight
[b] As found in Appendix E, Table E-1.
[c] Tolerance Interval, specified to contain 99% of commercial variety population with 95% confidence, negative limits set to zero.
[d] Range of values found in published literature for cotton varieties. [1]Cherry *et al*., 1978 (as % oil); [2]Cherry, 1983 (as % lipid); [3] NRC, 1982 (fuzzy seed); [4] Belyea *et al*., 1989; [5] NRC, 2001 (fuzzy seed); [6] Shenstone and Vickery, 1961 (as % oil); [7] Lawhon *et al*., 1977 (as g/16gN defatted flour)

VII.D. Overall Conclusions for Phenotypic Evaluation

A thorough phenotypic characterization of MON 88913 was performed comparing multiple phenotypic characteristics, including 11 characteristics during plant growth and development, 20 characteristics from plant mapping, four characteristics from boll/seed measurements, six boll and fiber quality characteristics, multiple seed germination regimes, eight reproductive morphology characteristics, and 69 compositional components. In addition, observational data on the presence and any differential response to biotic (pests and disease) and abiotic stressors were collected. These measurements are well known to cotton breeders and can provide supplementary data to assess plant pest potential.

Information was used to assess whether the presence of the DNA insert or the CP4 EPSPS protein altered the plant pest characteristics of MON 88913 compared to MON 88913(-). Agronomic data were also provided to support the concept of equivalence and familiarity as it relates to pest potential, and compositional data was provided to indicate whether levels of nutrients, antinutrients, toxicants or other components of MON 88913 are altered relative to the appropriate control and to commercial conventional cotton.

The overall conclusions from this extensive phenotypic characterization were that there are no biologically meaningful differences in terms of pest potential between MON 88913 and MON 88913(-) and the phenotype of cotton has been changed only with respect to the Roundup Ready trait.

VIII. Factors Influencing an Ecological Assessment of Roundup Ready Flex Cotton

This section provides relevant information regarding the introduced trait, interactions with pest and nonpest organisms, potential to become a weed, gene flow potential, agronomic practices, occurrence of weeds and their control in conventional and herbicide-tolerant cotton, and volunteer management practices that have been used to conduct an environmental assessment of MON 88913.

MON 88913 provides enhanced reproductive tolerance when compared to Roundup Ready cotton. This improved tolerance of MON 88913 allows a wider window for over-the-top glyphosate herbicide applications. Over-the-top applications into or over the canopy enhances weed control by increasing foliar coverage of the weeds, minimizing escapes, and reducing the potential for applying sublethal rates, which is possible when using post-directed equipment. The wider over-the-top application window will improve overall weed control efficacy by ensuring that the appropriate herbicide rate will be delivered to the target weed. Farmers currently utilize Roundup Ready cotton on approximately 59% of U.S. acres (USDA-NASS, 2003b) due to its economic, weed control and convenience benefits. It is expected that MON 88913 will rapidly replace the majority of these Roundup Ready cotton acres. Therefore, from an ecological perspective, the transition to MON 88913 is not expected to alter either the crop rotational practices or volunteer control measures currently being utilized by U.S. cotton growers today.

VIII.A. Characterization of the Trait

VIII.A.1. Safety and Nutrition

Five Roundup Ready crops that produce CP4 EPSPS have been reviewed by regulatory agencies and cleared for environmental release in one or more countries around the world. These products are Roundup Ready canola, Roundup Ready corn, Roundup Ready cotton, Roundup Ready soybean and Roundup Ready sugar beet. Extensive compositional data demonstrate that these crops are nutritionally equivalent to their conventional counterparts. Likewise, the safety assessment of the CP4 EPSPS protein produced in MON 88913 includes a protein characterization demonstrating the lack of similarity to known allergens and toxins and the long history of safe consumption of similar proteins. In addition, data confirm the CP4 EPSPS protein digestibility *in vitro*, and the lack of acute oral toxicity in mice.

Similar to other Roundup Ready crops, the composition of field-generated cottonseed from MON 88913 demonstrates that the levels of key nutrients and other components are within the same range as conventional cotton. There were few statistically significant differences between MON 88913 and MON 88913(-) and the range of values for all

components associated with the statistically significant differences were found to fall within the 99% tolerance interval for the conventional cotton varieties with the exception of moisture in a combined site comparison. It is unlikely that any of the minor differences detected are biologically meaningful or would contribute to pest potential. Cottonseed from MON 88913 is therefore considered to be nutritionally equivalent to cottonseed of conventional cotton.

VIII.A.2. Interactions with Pest and Nonpest Organisms: Field Observations and Change in Toxicants

The CP4 EPSPS protein contained in MON 88913 and other Roundup Ready crops is similar to the native EPSPS protein that is ubiquitous in plant and microbial tissues in the environment. Therefore, based on this history of occurrence, the EPSPS protein is not expected to possess biological activity towards nonpest organisms. Even though the likelihood of hazard is low for the CP4 EPSPS protein, a number of researchers have conducted laboratory investigations with different types of arthropods exposed to Roundup Ready crops containing the CP4 EPSPS protein (Goldstein, 2003; Boongird et al., 2003; Jamornman, et al., 2003; Harvey et al., 2003). Representative pollinators, soil organisms, beneficial arthropods and pest species were exposed to tissues (pollen, seed, and foliage) from Roundup Ready crops that contain the CP4 EPSPS protein. These studies, although varying in design, all reported a lack of toxicity observed in various species exposed to Roundup Ready crops producing the CP4 EPSPS protein (Nahas et al., 2001; Dunfield and Germida, 2003, Siciliano and Germida 1999).

The lack of toxicity is further supported by field experimentation conducted on biotechnology-derived crops producing the CP4 EPSPS protein. Diversity and abundance of Collembola was no different between Roundup Ready soybeans and conventional soybeans grown under the same management systems (Bitzer et al., 2002). Other studies on registered Roundup Ready soybeans under various weed management systems concluded that there was no apparent direct effect of the Roundup Ready trait on arthropods, although weed management and phenotypic differences (plant height or maturity) associated with plant variety influenced arthropod populations (Jasinski et al., 2003; McPherson et al., 2003; Buckelew et al., 2000). A similar lack of effect on arthropods is expected for MON 88913.

In addition to the lack of observed toxicity of the CP4 EPSPS protein, the compositional analysis of MON 88913 (Section VII.C; Appendix E), found that there were no significant differences between MON 88913 and MON 88913(-) for the toxicants (aflatoxins B1, B2, G1, and G2), gossypol (free and total), and there were no significant differences in the combined site analysis for the antinutrient cyclopropenoid fatty acids (malvalic acid, sterculic acid, and dihydrosterculic acid). A significant difference in malvalic acid and sterculic acid occurred at a single site, which did not occur at the other three locations. These observed differences are unlikely to be biologically meaningful because the range of values for these analytes were found to fall within the 99% tolerance interval for the commercial varieties planted in the same field trials as MON 88913 and

MON 88913(-). Therefore there is no reason to anticipate that MON 88913 would impact nonpest organisms beyond that expected for other cotton plants.

VIII.B. Ecological Characterization of MON 88913

VIII.B.1. Potential for Roundup Ready Flex Cotton MON 88913 to Become a Weed

Commercial *Gossypium* species in the U.S. are not considered as weeds and are not effective in invading established ecosystems. Cotton is not considered to have weedy characteristics in the U.S. It does not possess any of the attributes commonly associated with weeds, such as long soil persistence, the ability to invade and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation. It is recognized that in some agricultural systems, cotton can volunteer in a subsequent rotational crop. However, volunteers are easily controlled through tillage or use of appropriate herbicides. In the continental U.S., wild populations of *Gossypium* species and some feral populations of cultivated variants of *G. hirsutum* exist in south Florida. However, the range is limited due at least in part to the fact that cotton does not survive as a perennial in those areas where freezing conditions occur and volunteers are easily managed in rotational crops using tillage and herbicides registered for control of cotton.

There is little probability that MON 88913 or any *Gossypium* species crossing with it could become a problem weed. In the comparative studies between MON 88913 and MON 88913(-), dormancy, germination, phenotypic and flower morphology characteristics were evaluated for changes that would impact plant pest potential and, in particular, plant weed potential (Section VII., Appendix C.). Based on these data, there was no evidence to suggest that MON 88913 has a higher likelihood to become a weed than conventional cotton. Furthermore, monitoring of field trial plots containing MON 88913 after harvest has not revealed differences in survivability or persistence relative to other varieties of cotton (Appendix A). As mentioned previously, MON 88913 is intended to replace the currently commercialized Roundup Ready cotton product. After seven years of U.S. commercial use of Roundup Ready cotton, there are no reports that cotton with this trait has become a problem weed.

VIII.B.2. Potential Impact of MON 88913 on Nonpest Organisms

During the phenotypic field trials at 14 locations in 2002 (Section VII.), each field site was rated at four times during the season for specific insect pests, diseases and abiotic stressors. The purpose of these trials was to assess whether the plant-disease or plant-insect interactions of MON 88913 were altered compared to MON 88913(-). Fourteen insect categories (species or group), four disease categories and ten abiotic stressors were evaluated. Out of 106 insect observations, only one site reported a difference at one of the four observation times in insect susceptibility between MON 88913 and MON 88913(-). This difference was not observed in other replications or at the other

observation times or locations, and so it is unlikely to be of biological importance. Out of seven disease and 38 abiotic stressor observations, no differences were detected between MON 88913 and MON 88913(-). These results support the conclusion that ecological interactions have not been changed in MON 88913 relative to other cotton.

VIII.C. Potential for Pollen-Mediated Gene Flow

VIII.C.1. Biogeography

As discussed in Section II., only two 'wild' *Gossypium* species related to cultivated cotton are known to be present in the U.S., *G. thurberi* Todaro, which is known in Arizona, and *G. tomentosum* Nuttall ex Seeman, which is endemic to Hawaii. Only *G. tomentosum* is considered to be capable of crossing with domesticated cotton and produce fertile offspring. Importantly, the Roundup Ready trait would not be expected to confer a selective advantage to, or enhance the pest potential of, progeny resulting from such a cross if it were to occur. However, domesticated cotton is not grown commercially in Hawaii, with the exception of counter-season breeding nurseries where appropriate isolation distances are employed. Thus, the potential for gene flow to these wild relatives is limited. Feral populations of cultivated *G. hirsutum* and 'wild' populations of *G. hirsutum* race '*yucatanense*' are known to occur in South Florida and Puerto Rico [redacted] personal communication) which would be capable of crossing with cultivated cotton, but are not known to exist in cotton growing areas.

VIII.C.2. Vertical Gene Flow

Assessment of Cross-Pollination in Cotton

Although natural crossing can occur, cotton is normally considered to be a self-pollinating crop (Niles and Feaster, 1984). There are no morphological barriers to cross-pollination based on flower structure. However, the pollen is heavy and sticky and transfer by wind is limited. Pollen is transferred instead by insects, in particular by various wild bees, bumble bees (*Bombus* sp.), and honeybees (*Apis mellifera*).

Early reports had indicated potentially high levels of cross-pollination between cotton plants in close proximity (McGregor, 1976: Webber (1903), Ricks and Brown (1916), Simpson (1954), and Simpson and Duncan (1956). The crop conditions reflected in these older reports may no longer exist. More recent cotton literature shows that the frequency of cross-pollination decreases with distance from the pollen source. McGregor (1976) traced movement of pollen by means of fluorescent particles and found that, even among flowers located only 150 to 200 feet from a cotton field that was surrounded by a large number of bee colonies to ensure ample opportunity for transfer of pollen, fluorescent particles were detected on only 1.6% of the flowers. In a 1996 study with various field designs, Llewellyn and Fitt (1996) also found low levels of cross-pollination in cotton. At one meter from the source they observed cross-pollination frequencies of 0.15 to 0.4%, decreasing to below 0.3 % at 16 meters from the source.

Umbeck et al., (1991) used a selectable marker to examine cross-pollination from a 30 x 136 meter source of biotechnology-derived cotton. Cross-pollination decreased from five to less than one percent from one to seven meters, respectively, away from the source plot. A low level of cross-pollination (less than one percent) was sporadically detected to the furthest sampling distance of 25 meters. Berkey et al., (2002) reported that cross-pollination between fields separated by a 13 foot road decreased from 1.89% in the row nearest the source to zero percent in the 24th row. For the sake of comparison, the isolation distances for foundation and certified cotton seed are 1320 and 660 feet, respectively (7CFR § 201.76).

Based on information previously submitted by Monsanto, the USDA stated in the environmental assessment documents for Bollgard and Roundup Ready cotton that the "potential for gene introgression from genetically engineered cotton lines into wild or cultivated sexually compatible plants is very low" (USDA, 1995a, 1995b). Importantly, the environmental consequences of pollen transfer from MON 88913 to other cotton or related *Gossypium* species is considered to be negligible because of limited movement of cotton pollen, the safety of the introduced protein, and lack of any selective advantage by the Roundup Ready trait that might be conferred on the recipient feral cotton or wild relatives.

<u>*Gene Flow to Wild Relatives*</u>

Based on cytological evidence, seven genomic types, A through G, many with subtypes, have been identified for the genus *Gossypium* (Endrizzi et al., 1984). The domesticated species *G. hirsutum* and *G. barbadense* are allotetraploid (AADD, 2n=4x=52), while *G. thurberi* is a diploid (DD, 2n=2x=26), and *G. tomentosum* is an allotetraploid (AADD, 2n=4x=52). *G. tomentosum* is considered to be capable of crossing with domesticated cotton to produce fertile offspring; however, cotton is not grown commercially in Hawaii and thus the potential for gene flow to these wild relatives is limited. Any potential gene exchange between *G. thurberi* and domesticated cotton, if it were to occur, would result in triploid (ADD, 3x=39), sterile plants because *G. hirsutum* and *G. barbadense* are allotetraploids (AADD, 2n=4x=52) and *G. thurberi* is a diploid (DD, 2n=2x=26). Such sterile hybrids have not been observed to persist in the wild. Fertile allohexaploids (6x=78) have not been reported in the wild.

<u>*Gene Flow to Feral Cotton*</u>

No feral populations (domesticated plants capable of surviving outside of cultivation) of *G. barbadense* have been found in the U.S. and thus only cultivated *G. barbadense* plants would be available for cross pollination by *G. hirsutum*. Seed production fields are segregated from other cotton fields to prevent cross-pollination. If cross-pollination were to occur, it almost certainly would involve plants producing seeds intended for processing rather than planting because seed production fields are isolated from commercial cotton fields. Therefore, any such escape of genes into *G. barbadense* would be very short-lived and of no significance. This would also be true if genes were to be transferred from *G. hirsutum* into another strain of cultivated *G. hirsutum*. As noted above, wild and feral *G. hirsutum* grows in southern Florida and, while it is possible that genes could escape to a feral *G. hirsutum,* it is unlikely because there is no commercial cotton production within

several hundred miles of this area. Escape of genes to *G. tomentosum* in Hawaii would be possible. However, this is also not likely to occur, because there is no commercial cotton production on these islands with the exception of counter-season breeding nurseries where isolation distances are employed.

VIII.C.3. Transfer of Genetic Information to Species With Which Cotton Cannot Interbreed (Horizontal Gene Flow).

Monsanto is not aware of any reports regarding the unaided transfer of genetic material from cotton species to other species with which cotton cannot sexually interbreed.

VIII.D. Agronomic Practices

VIII.D.1. Introduction

This section provides a review of U.S. agronomic practices in cotton and the anticipated environmental consequences of commercialization of Roundup Ready Flex cotton, MON 88913. Included is a discussion of current cotton production practices, weed occurrence and their management, cotton rotational crops and volunteer cotton management. An update on the current use of Roundup Ready cotton is provided as well as the expected use of MON 88913.

As with other crops, common steps exist in cotton production which include fertilizer placement, seedbed preparation, planting, the management of insects, weeds and diseases during the season, and harvest. Although the length of the season may vary with geography, the production cycle and techniques used are fairly consistent among geographies and between the upland and pima cotton types.

Weeds cause significant losses and require careful management by the grower as they interfere with the cotton through their competition for available resources including water, nutrients and light. They also impede harvest and have a negative economic impact on the grower by reducing cotton lint yields and lint quality. Economically damaging weeds in cotton include both annual and perennial, grasses, broadleaf and sedge species.

Methods of weed control include cultural, mechanical, biological and chemical methods. In cotton, chemical weed control is widely used along with the use of tillage (e.g., seed bed preparation, tillage) and cultural (e.g., crop rotation, field selection) methods. Currently, a wide variety of herbicides are available and used in cotton production. Roundup agricultural herbicides have been used successfully in combination with Roundup Ready cotton since its commercial introduction in 1996.

Volunteer cotton primarily occurs in cotton following a previous cotton crop. This is due to a predominant use of continuous cotton compared to the minor usage of a rotation to

corn or soybean. In cotton and other rotational crops, growers have traditionally controlled volunteer cotton using a combination of mechanical and chemical methods.

VIII.D.2. U.S. Cotton Production

Cotton production is complex and efficiently managing production practices such as soil fertility, planting, pest management, irrigation and harvest is difficult (Oosterhuis and Jernstedt, 1999). Cotton production in the U.S. is limited primarily by climate. Cotton is a warm-season plant and successful production requires 200 frost-free days, and more than 120 days above 15°C (Waddle, 1984). Cotton is generally produced in the U.S. below 36° N latitude. However, cultivation has expanded into slightly more northern areas of the Mississippi valley, Oklahoma and California (Waddle, 1984). Aside from temperature, the most influential climatic factor impacting cotton agronomic practices is moisture.

Cotton is not sensitive to any particular soil type, provided sufficient nutrition, adequate temperature and moisture are available. A minimum of ~20 inches of moisture is generally required, but at least twice that amount of rainfall is generally expected for cotton production in the humid Southeast and Mississippi Delta (Midsouth). In some coastal areas of the South and Southeast, rainfall can be excessive and can actually limit cotton production. In other areas with less rainfall, seasonally strong thunderstorm activity and/or hail, stripper cotton varieties are often produced (such as in the high plains of Texas). Whereas "picker" cotton varieties produce large open bolls amenable to hand picking and harvesters, "stripper" cotton varieties produce a 'tight' boll that is more resistant against yield loss under storm and hail conditions.

Cotton grown in the U.S. west of about 100° W longitude is likely to suffer from significant moisture stress during the season, and requires irrigation to maximize yield. Daily water use in cotton is as much as 0.45 inches per day across regions of the Southwest, with maximum use during July and August. The average daily water use in the San Joaquin valley of California is 0.3 inches per day during this period of peak use (Hake et al., 1996c). Supplying the necessary moisture to meet these seasonal needs is a primary goal of an irrigation program. The amount of water needed can be determined by a number of factors: rooting depth, soil moisture-holding capacity, available moisture-holding capacity, the current moisture level, and uniformity of water application (Hake et al., 1996c). In addition, the amount of water needed will depend on the stage of crop development, as overwatering can lead to anaerobic stress, which can cause yield loss during the flowering period. In certain arid regions where irrigation is cost-prohibitive, non-irrigated dryland cotton is grown. This is primarily in areas obtaining ≤ 20 inches of rainfall, and where other crops are not practical (Waddle, 1984). Under these conditions, reduced yield are expected, and reduced planting densities, can be employed to conserve soil moisture.

Based on climatic and moisture requirements, cotton is currently produced in 17 states across the southern U.S., extending from Virginia south and west to California (the U.S.

cottonbelt). In 2002, nearly 14 million acres of cotton were planted in this area, producing more than 17 million bales of cotton (USDA-NASS, 2003a). In the U.S., upland cotton is the most commonly cultivated species with 13.7 million acres planted across the cottonbelt in 2002, while a much smaller amount (244,000 acres) of pima cotton is produced in western regions including Arizona, California, New Mexico, and Texas (USDA-NASS, 2003a). With 5.8 million acres planted, Texas produces more cotton than any other state, producing five million bales in 2002. Other states producing over one million bales in 2002 included Arizona, California, Georgia and Mississippi.

A consolidation of the four USDA-ERS estimated cotton growing regions in the United States is shown in Table VIII-1. The Southeast region includes the states of North Carolina, South Carolina, Georgia and Alabama, and is generally a mix of small and larger farms. The Midsouth region includes the states of Missouri, Tennessee, Arkansas, Louisiana and Mississippi and encompasses the area historically known as the Mississippi Delta. The Southwest region includes the states of Texas, New Mexico, Oklahoma and Kansas. This region has the most cropland of the cotton growing regions, but has a much drier climate compared to the Southeast and Midsouth regions. The West region includes the states of Arizona and California, and has the largest share of large family and non-family farms.

The Southeast and Midsouth had fairly equivalent operation costs and levels of production in 2000 and 2001, as seen in Table VIII-1. Total gross value of the cotton crop, a combination of the gross value of the cotton lint and the gross value of the cottonseed, are within a few dollars on a per acre basis for both regions in the years shown. Although total operating costs were marginally higher in the Southeast compared to the Midsouth, pesticide expenditures showed a slightly higher trend in the Midsouth compared to the Southeast. Lint and cottonseed yields from both regions were very similar in 2000 and 2001. The majority of cotton grown in the Southeast and Midsouth is not irrigated. Eighty nine percent of the acres in the Southeast and 70% of the acres in the Midsouth are in dryland production (USDA-NASS, 2003a).

The Southwest has the most cropland of the cotton growing regions, but in 2000-2001 the Southwest had the lowest average cotton lint and cottonseed yield of the four regions. Lint and cottonseed yields in the Southwest in 2000 and 2001 were approximately half of those in the Southeast and Midsouth, most likely due to the dry climate conditions in these states. The Southwest had the least total operating costs of the four regions in 2000 and 2001. Although the Southwest is in a dryer climate that either the Southeast or Midsouth, the majority of the acres are in dryland production, with only 30% of the acres irrigated (USDA-NASS, 2003a).

The West region had the highest total cotton gross value in both 2000 and 2001, and the highest cotton lint yield in 2000 and 2001 compared to the other regions. Total operating costs were also higher in the West compared to the other regions in 2000 and 2001. This was at least partially due to a cost of more than $40 per planted acre for purchasing irrigation water, which was included in the total operating costs for the West (USDA-NASS, 2003a). More than 70% of the cotton acres in the West are irrigated.

Table VIII-1. U.S. Cotton Production: Value and Production Costs by Region.

	Southeast[1]		Midsouth[1]		Southwest[1]		West[1]		All U.S.	
	2000	2001	2000	2001	2000	2001	2000	2001	2000	2001
	Dollars per Planted Acre									
Gross Value - Cotton Lint	389.76	308.58	379.77	303.13	159.53	94.08	661.76	346.50	324.33	222.60
Gross Value - Cottonseed	50.48	63.96	59.50	62.20	29.50	25.20	108.08	87.60	50.85	48.80
Gross Value - Total	440.24	372.54	439.27	365.33	189.03	119.28	769.84	434.10	375.18	271.40
Total Operating Costs	300.52	329.95	278.54	302.14	161.42	171.11	485.98	470.70	269.38	284.24
Pesticide Costs*	68.48	68.43	79.50	79.73	25.67	25.67	92.07	87.46	58.32	59.25
Value of Production Less Operating Costs	139.72	42.59	160.73	63.19	27.61	-51.83	283.86	-36.60	105.80	-12.84
Cotton Yield: Pounds per Planted Acre	672	834	690	835	301	336	1034	990	569	636
Price: Dollars per Pound	0.58	0.37	0.55	0.37	0.53	0.28	0.64	0.35	0.57	0.35
Cottonseed Yield: Pounds per Planted Acre	1262	1599	1190	1555	590	630	1544	1460	1017	1220

[1]USDA-ERS (2003b) estimates: Southeast includes NC, SC, GA and AL; Midsouth includes average of TN, AR, MS, and LA together with the costs of MO.
Southwest includes TX, NM, OK and KS; West includes CA and AZ.
*Pesticide costs are included in the Total Operating Costs.
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VIII.D.3. Production Considerations

Pre-Season
Decisions made prior to planting regarding crop rotation, soil fertility, variety selection and other factors impact the entire growing season. The rotation of cotton with other crops should be an integral part of a farm management program. Ideally, cotton should be rotated with other row crops on a regular basis to maintain soil productivity and reduce the incidence of various weed, insect pests or diseases. However, as discussed later, cotton is most often replanted to cotton rather than rotated with other crops (USDA-ERS, 2003a).

Maintaining optimum crop nutrition is critical in achieving high yields and quality in cotton. The type and level of nutrition needed varies with the soil type and stage of crop development. For most cotton across the cottonbelt, the essential mineral nutrients of concern are the macronutrients nitrogen, phosphorus and potassium; the secondary nutrients magnesium, calcium and sulfur; and the micronutrients boron, copper, chlorine, iron, molybdenum, manganese, and zinc. The management of nitrogen for cotton is particularly critical because, in addition to the effects of deficiencies, excesses can promote excess vegetative growth (at the expense of fruiting) and delayed maturity which results in lower yields. Pre-season soil testing results together with previous cropping and fertilization history help determine fertilizer needs for the upcoming crop. In Southwestern and Western portions of the cottonbelt monitoring soil salinity is of additional importance (Silvertooth et al., 1999).

In terms of varietal selection, yield has traditionally been the most important factor considered by growers across the cottonbelt (Kerby et al., 1996). However, today, growers also need to consider fiber quality (length, strength, micronaire, etc.) due to its increasing importance, along with seedling vigor, maturity and a number of other factors. In general, cotton varieties are classified into three maturity groups: short-, medium-, or long-season varieties. The short season, more determinate plants are planted in northern portions of the cottonbelt with longer-season or more indeterminate varieties planted in the south. Growers in areas of western Texas and Oklahoma have tended to select 'stripper' or 'stormproof' varieties versus 'picker' cotton used in other areas due to the need for resistance to adverse weather conditions later in the season. In most recent years the availability of biotechnology-enhanced products with *lepidopteran* insect protection and herbicide tolerance has also become a key consideration in variety selection (Silvertooth et al., 1999).

Planting and Early Season
Cotton should be planted into prepared seedbeds that are firm, warm and moist because cool and wet conditions during the early part of the growing season can adversely affect development. The yield potential of a cotton crop is determined in the first 30 to 40 days after seed is placed in the ground (Deterling and El-Zik, 1982). Due to its tropical origins, temperatures below 60° F will slow germination, emergence and seedling growth. During the first 60 to 100 hours of germination, the radicle tip is easily damaged by chilling or lack of oxygen in the soil. If the tip is killed, a shallow system of secondary

roots develops that makes the plant more susceptible to moisture stress later in the season (Hake et al., 1996a, b). For this reason it is generally recommended to not plant cotton until soil temperatures at seeding depth are at 64° F or higher at 8 a.m. on three consecutive days, with a favorable five-day forecast (Silvertooth et al., 1999; Deterling and El-Zik, 1982).

In calculating a proper seeding rate, seed per row foot is the preferred method as this translates well from variety to variety and with row spacing. Seed sizes in cotton can vary greatly among cultivars, with some varieties having less than 4,000 seeds per lb. while other varieties may have over 7,000 seeds per lb. Row spacings vary across the cottonbelt but generally range from 30 to 40 inches with a number of variations including skip- and narrow-row cotton. A good final plant population target is two to three plants per row foot. Most cotton seed sold commercially is treated with a fungicide to protect the germinating seed and seedlings from fungal diseases.

Once emerged, the cotton plant goes through a period of slow growth and development before entering phases of rapid vegetative and reproductive growth. Under favorable conditions, the cotton plant will send a taproot downward for several days without branching. It may reach a depth of nine inches by the time that the cotyledons have emerged from the soil (Deterling and El-Zik, 1982). Soil type, texture, moisture and aeration determine how deep taproots will penetrate with normally about one-half of the total root length confined to the top two feet of soil. The basic root system normally is in place by the time the plant begins blooming eight to ten weeks after planting. It is during this early period of slow development that cotton must be protected from damaging weed, insect and disease pests (Bryson et al., 1999; Hake et al., 1996b).

Above ground, the developing cotton plant has a prominent, erect stem consisting of a series of nodes and internodes. In cotton, each new node with extended leaf develops three-eights of a turn above the preceding node in a spiral pattern. This arrangement provides for minimal self shading of lower leaves as new nodes develop. A terminal bud at the top of the plant controls the upward pattern of the stem, leaf and branch development. If damaged by hail, insects or mechanical operations, the entire growth sequence of the plant can be detrimentally affected, resulting in irregular branching and growth delays.

Mid-Season

After early development, the next critical stage in the development of a cotton crop is rapid vegetative growth that includes the initiation of the first 'squares' or floral buds. Eventually, these will develop into the plant's first bolls. Overall, the developing cotton plant will set from three to eight 'vegetative' (monopodial) branches prior to the establishment of reproductive or 'fruiting' (sympodial) branches. As indicated, vegetative branches will produce primarily vegetation, mostly leaves, and they are nearly upright. The leaves of the cotton plant may vary in size, texture, hairiness and green color depending on the variety. Weather conditions and cultural practices such as fertilization and irrigation can also influence the size, thickness and color of the leaves (Deterling and El-Zik, 1982).

Fruiting development generally begins with the formation of fruiting branches on nodes five through seven. In general, short-season varieties will set their first fruiting branch lower on the plant at the fourth or fifth node compared to long-season varieties which may not set the first reproductive branch until node eight. In addition to genetic differences, plant population, temperature and environmental stress also influence the location of the first fruiting branch.

Retention of the maximum number of squares and resulting bolls, especially the first bolls set on the plant, is critical to achieving maximum cotton yield. As cotton transitions into reproductive growth it is important to monitor and manage the crop's growth and development. The first three 'positions' on each reproductive branch are the key sites for fruiting and will account for the vast majority of the plant's yield. Further, the first position, or squares nearest the main stem, will account for over 50% of the total lint produced. The second series of squares (e.g., two positions away from the stalk) account for another one-third or more of the harvest. Squares further out on each reproductive branch (e.g., three or more) produce 15% or less of the final number of mature bolls (Deterling and El-Zik, 1982).

The modern grower or crop consultant currently uses a number of tools during mid-season to monitor and manage cotton plant growth. Although each will not be discussed in detail here, the grower may monitor any one or more of the following parameters: (1) plant height; (2) frequency of mainstem nodes; (3) maximum internode distance; (4) height-to-node ratios; and (5) square or fruit retention. In general, the cotton grower is seeking to favor reproductive growth at the expense of vegetative growth. Available options to influence cotton plant growth include the use of a plant growth regulator such as mepiquat chloride, fertility management (primarily nitrogen and potassium), and irrigation. Also, insect pest control is important at mid-season, as populations above economic thresholds can dramatically decrease both square and immature boll retention.

Late Season and Harvest

As the end of the season approaches, the yield is established and management efforts shift to protecting the crop yield and quality. Inputs such as irrigation, fertilizer and insecticide sprays generally stop when the crop shows pronounced decreases in growth, flowering and boll retention. The stage in cotton when vegetative growth ceases is generally referred to as 'cut-out'. The best method of estimating cut-out is to monitor the number of nodes above the highest first position white flower. When the 'nodes above white flower' decline to four or five, cut-out has been reached.

Effective defoliation is an essential step in the overall process of harvesting high quality cotton lint with the producer seeking to accomplish a complete, quick and efficient defoliation from a single application of defoliant. Successful defoliation in cotton depends on a number of factors including: (1) plant-water status; (2) nitrogen fertility status; (3) weather conditions; and (4) the chemical defoliant(s) (Silvertooth et al., 1999). Among these, the defoliation material(s) selected may be considered the most influential due to the large number of cotton products labeled for cotton defoliation.

However, for good defoliation to occur, the cotton plant should be low (but not deficient) in moisture and nitrogen. Weather, especially temperature, can also have a large impact on the efficacy and performance of the defoliant. As temperatures increase, the activity of most defoliants also increases.

Control of weeds in a cotton crop before harvest is essential because weeds compete with the crop for limited resources (Ross and Lembi, 1985; Wilcut et al., 2003) and failure to control weeds can result in decreased yields and reduced crop quality (Wilcut et al., 2003; Hayes et al., 2001). In addition, weeds present at cotton harvest reduce the efficiency of the mechanical harvest of the crop and can reduce both the quality and value of the lint because of staining by vegetation.

VIII.D.4. Occurrence of Weeds in Cotton Production

Weed control in cotton is essential to maximize both cotton fiber yield and quality. In contrast to most crops including corn and soybean, cotton emergence and above ground growth is relatively slow during the first few weeks after planting. The slow early growth habit of cotton does not permit the crop to aggressively compete against often more rapidly developing weed species. This is especially true under cool weather or adverse growing conditions which often prevail during the spring. Various weed-crop competition studies have demonstrated that the control of weeds during the first four to eight weeks after cotton planting is critical as weeds compete against the crop for water, nutrients, light and other resources necessary for growth (Bryson et al., 1999). Currently in the U.S., weeds are controlled through the integrated use of various cultural, mechanical and chemical control methods (Buchanan, 1992; Ridgway et al., 1984).

The occurrence of weeds in cotton is well documented. Since 1971, the Southern Weed Science Society has published its weed survey of the southern states as a part of their Research Report. The most recent survey of the most common and most troublesome weeds in cotton was published in 2001 proceedings (SWSS, 2001). Tables VIII-2 to VIII-5 provide summaries of the most troublesome weeds in cotton in each state by taxonomic family. States are grouped by the four major cotton growing regions discussed earlier (Southeast, Midsouth, Southwest and West), except the Southeast includes Florida. In addition, University researchers meeting at the annual Beltwide Cotton Conferences meeting have regularly estimated yield losses in cotton caused by grass, sedge and broadleaf weed species. These estimates are provided in Tables VIII-6 and VIII-7.

Southeastern U.S.

As summarized by Murray, Verhalen and Tyri for the 15-year period 1970 to 1985, the Amaranthaceae, Asteraceae (Compositae), Convolvulaceae, Cyperaceae, Euphorbiaceae, Poaceae (Gramineae), Leguminoseae and Malvaceae families appear as "the 10 most troublesome (and common) weeds" of the Southeast states (Murray et al., 1992). The authors reported that over that period, "no apparent trend existed for reduced numbers of families over time". In reviewing the data shown in Table VIII-2 the same conclusion

can be reached for the 2001 data (SWSS, 2001). Each of the weed taxonomic families cited above continues to be represented by weed species in 2001, in addition to Rubiaceae, present but not specifically cited in the quote above. Among the most prevalent weed species in Southeastern cotton were pigweed species (*Amaranthus* sp.), morningglory species (*Ipomoea* sp.), nutsedge species (*Cyperus* sp.), tropic croton (*Croton glandulosus*), sicklepod (*Cassia obtusifolia*), and bermudagrass (*Cynodon dactylon*). In terms of yield reduction, *Amaranthus*, *Ipomea*, *Senna* and *Cyperus* species were among the most economically damaging (Byrd Jr., 2003) (Tables VIII-6 and VIII-7).

The only trend cited by the authors for 1970 to 1985 was a trend for more representatives per family over time, specifically Poaceae, with seven members. In examining the results for 2001, this trend appears to have been largely reversed in subsequent years, as only two species, Texas panicum and bermudagrass, were cited as troublesome Poaceae weed species across the Southeastern U.S. in cotton production. This is likely due to the development and extensive use of Roundup Ready cotton across the Southeastern U.S. and the general susceptibility of grass weed species to glyphosate, the active ingredient in Roundup agricultural herbicides.

Table VIII-2. The Ten Most Troublesome Weeds Present in Cotton in the Southeastern U.S.[1]

North Carolina	South Carolina	Georgia	Florida	Alabama
Amarathaceae Palmer amaranth	**Amarathaceae** Palmer amaranth	**Amarathaceae** Pigweed sp.	**Amarathaceae** Pigweed sp.	
Asteraceae Common Cockelbur	**Asteraceae** Common Cockelbur			**Asteraceae** Common Cockelbur
		Commelinaceae Dayflower sp.		
Convolvulaceae Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.
Cyperaceae Nutsedge sp.	**Cyperaceae** Nutsedge sp.	**Cyperaceae** Nutsedge sp.	**Cyperaceae** Nutsedge sp.	**Cyperaceae** Nutsedge sp.
Euphorbiaceae Tropic croton	**Euphorbiaceae** Spotted spurge	**Euphorbiaceae** Wild Poinsettia Tropic croton	**Euphorbiaceae** Wild Poinsettia	**Euphorbiaceae** Tropic croton Spurge sp.
Leguminoseae Sicklepod	**Leguminoseae** Sicklepod Coffee senna Cowpea	**Leguminoseae** Sicklepod	**Leguminoseae** Sicklepod Florida beggarweed	**Leguminoseae** Sicklepod Coffee senna
Malvaceae Velvetleaf Spurred anoda	**Malvaceae** Velvetleaf			**Malvaceae** Velvetleaf
Poaceae Bermudagrass		**Poaceae** Texas panicum Bermudagrass	**Poaceae** Texas panicum Bermudagrass	**Poaceae** Bermudagrass
Polygonaceae Smartweed sp.			**Polygonaceae** Smartweed sp.	**Polygonaceae** Smartweed sp.
	Rubiaceae Florida pusley	**Rubiaceae** Florida pusley	**Rubiaceae** Forida pusley	

[1]From survey published by SWSS, 2001.

U.S. Midsouth

Historical survey results from the Midsouth from 1970 to 1985 cite the families Amaranthaceae, Asteraceae (Compositae), Bignoniaceae, Convolvulaceae, Cyperaceae, Euphorbiaceae, Poaceae (Gramineae), Leguminoseae, Malvaceae, Polygonaceae and 'Vines' among the most troublesome in cotton production (Murray et al., 1992) as seen in the 2001 summarized estimates in Tables VIII-6 and VIII-7 and below in Table VIII-3. Each of these families continue to be represented as the most troublesome weeds in cotton. Among the most prevalent and economically damaging species in Midsouth cotton were pigweed species (*Amaranthus* sp.), morningglory species (*Ipomoea* sp.), prickly sida (*Sida spinosa*), common cocklebur (*Xanthium strumarium*), johnsongrass (*Sorghum halapense*), bermudagrass (*Cynodon dactylon*), and nutsedges (*Cyperus* sp.) (SWSS, 2001; Byrd Jr., 2003; [redacted] personal communication, 2004).

Table VIII-3. The Ten Most Troublesome Weeds Present in Cotton in the U.S. Midsouth.[1]

Mississippi	Louisiana	Tennessee	Missouri	Arkansas[2]
Amarathaceae Pigweed sp.	**Amarathaceae** Pigweed sp.	**Amarathaceae** Palmer amaranth Smooth pigweed	**Amarathaceae** Palmer amaranth	**Amarathaceae** Palmer amaranth
Asclepiadaceae Honeyvine milkweed				
		Asteracea Common Cockelbur	**Asteracea** Common Cockelbur	
Bignoniaceae Trumpet creeper		**Bignoniaceae** Trumpet creeper		
Convolvulaceae Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.	**Convolvulaceae** Morningglory sp.
	Cyperaceae Nutsedge sp.	**Cyperaceae** Yellow nutsedge		**Cyperaceae** Yellow nutsedge
Euphorbiaceae Spotted spurge		**Euphorbiaceae** Spotted spurge	**Euphorbiaceae** Spurge sp.	
Leguminoseae Hemp sesbania	**Leguminoseae** Hemp sesbania Sicklepod			**Leguminoseae** Sicklepod
Malvaceae Prickly sida	**Malvaceae** Prickly sida Wild okra	**Malvaceae** Prickly sida Velvetleaf	**Malvaceae** Prickly sida Velvetleaf	**Malvaceae** Prickly sida Velvetleaf
Poaceae Bermudagrass Southern crabgrass	**Poaceae** Bermudagrass	**Poaceae** Bermudagrass	**Poaceae** Johnsongrass Bermudagrass Goosegrass	**Poaceae** Barnyardgrass Johnsongrass Crabgrass Broadleaf signalgrass
			Perennial vines	
Polygonaceae Redvine	**Polygonaceae** Redvine Penn. smartweed			

[1]From survey published by SWSS, 2001.

Southwestern U.S.

Historically, in the Southwestern states of Texas and Oklahoma weed species in the families Amarathaceae, Convolulaceae, Cyperaceae, Euphorbiaceae, Poaceae (Graminae), Malvaceae, Portulacaceae and Solanaceae have been historically been problems in cotton (Murray et al., 1992). In 2001, these families continued to be the source of a number of the most troublesome weed species along with members in the Asteraceae, Cucurbitacea and Pedaliacea families (Table VIII-4). As in other parts of the cottonbelt, pigweed, morningglory, johnsongrass, nutsedge and silverleaf nightshade (*Solanum elaegnifolium*) are common problems (SWSS, 2001; Byrd Jr., 2003)

Table VIII-4. The Ten Most Troublesome Weeds Present in Cotton in the Southwestern U.S.[1]

Oklahoma	Texas[2]
Amarathaceae Pigweed sp.	
Asteracea Common Cockelbur	**Asteracea** Woolyleaf bursage Texas blueweed
Convolvulaceae Morningglory sp. Field bindweed	**Convolvulaceae** Sharppod morningglory
	Cucurbitaceae Smell melon
Cyperaceae Yellow nutsedge	**Cyperaceae** Purple nutsedge Yellow nutsedge
	Euphorbiaceae Texasweed
	Lamiaceae Lanceleaf sage
Pedaliaceae Devil's claw	**Pedaliaceae** Devil's claw
Poaceae Red sprangletop Johnsongrass Texas panicum	
Solanaceae Silverleaf nightshade	**Solanaceae** Silverleaf nightshade

[1] From survey published by SWSS, 2001. Data not available for KS and NM.

[2] [redacted] Texas A&M University, personal communication 2004.

Western U.S.

Historically, in the Western U.S., the weed families which have historically been problems in cotton production (1973 to 1986) are Amaranthaceae, Chenopodiaceae, Asteraceae (Compositae), Convolvulaceae, Cyperaceae, Euphorbiaceae, Poaceae (Gramineae), Leguminosae, Malvaceae and Solanaceae (Murray et al., 1992). These weed families and species continue to be represented in more recent times as indicated in Tables VIII-5, VIII-6, and VIII-7. As estimated by percent reduction in cotton yields, researchers rank morningglory (*Ipomoea* sp.) and nutsedge (*Cyperus* sp.) species as the most economically damaging in Arizona while California researchers cite barnyardgrass, morningglory sp., nutsedge and nightshades (*Solanum* sp.) (Byrd Jr., 2003).

Table VIII-5. The Ten Most Troublesome Weeds present in Cotton Grown in the Western U.S.[1]

Arizona	California
Amarathaceae Palmer amaranth	
Convolvulaceae Morningglory sp.	**Convolvulaceae** Morningglory sp. Field bindweed
Cyperaceae Purple nutsedge Yellow nutsedge	**Cyperaceae** Purple nutsedge Yellow nutsedge
Poaceae Bermudagrass Johnsongrass Sprangletop	**Poaceae** Bermudagrass Johnsongrass
Portulacaceae Common purslane	
Solanaceae Wright groundcherry Silverleaf nightshade	**Solanaceae** Hairy nightshade Black nightshade

[1] McCloskey et al., 1998; Vargas et al., (2001); University of California, 2001.

Table VIII-6. Estimated Percent Reduction in Cotton Yields by Grass and Sedge Weeds by State in 2002.[1]

	Southeast					Midsouth					Southwest			West		
	NC	SC	GA	FL	AL	MS	LA	AR	TN	MO	TX	OK	NM	AZ	CA	Avg. % Loss
Grasses:	Percent Reduction															
Cynodon (Bermudagrass)	2	2	2	3	8	8	-	2	3	1	4	-	6	5	5	3.4
Digitaria (Southern Crabgrass)	1	-	2	3	5	2	3	6	1	5	1	3	-	-	-	2.1
Echinochloa (Barnyardgrass)	-	-	-	2	1	1	1	2	1	1	4	3	2	5	15	2.5
Eluisine (Goosegrass)	1	4	-	3	3	1	3	1	3	4	-	-	-	-	-	1.5
Panicum (Texas panicum)	1	3	12	3	5	7	4	1	1	1	5	5	-	1	-	2.8
Sorghum (Johnsongrass)	1	2	1	2	1	9	4	3	3	6	10	6	10	5	5	4.5
Sedges:																
Cyperus (Yellow nutsedge) (Purple nutsedge)	3	10	12	8	8	3	5	6	3	2	6	4	12	12	20	8.5

[1]Byrd Jr., 2003. Specific weed species not reported in Byrd Jr., 2003. Weed species added as representative of reported genus.

Table VIII-7. Estimated Percent Reduction in Cotton Yields by Broadleaf Weeds by State in 2002.[1]

	Southeast					Midsouth					Southwest			West		Avg. % Loss
	NC	SC	GA	FL	AL	MS	LA	AR	TN	MO	TX	OK	NM	AZ	CA	
	Percent Reduction															
Abutilon (Velvetleaf)	-	1	-	-	1	2	-	1	1	5	-	-	-	-	-	0.8
Amaranthus (Pigweed sp.)	35	38	25	5	6	15	7	15	10	6	12	13	2	5	7	13.4
Anoda (Spurred anoda)	-	-	-	-	1	1	1	2	2	3	1	-	12	-	-	1.5
Convolvulus (Field bindweed)	-	-	-	-	-	1	-	-	-	-	1	4	2	3	5	1.1
Croton (Tropic croton)	-	2	-	4	1	-	-	-	-	1	1	-	-	-	-	0.7
Euphorbia (Spurge sp.)	-	1	2	5	4	5	3	8	2	5	1	3	-	1	-	2.7
Ipomoea (Morningglory sp.)	35	12	25	10	17	27	10	25	10	22	12	14	25	15	19	16.0
Polygonum (Smartweed sp.)	1	1	-	3	-	3	-	-	1	3	-	-	-	-	-	0.8
Proboscidea (Devil's claw)	-	-	-	-	-	-	-	-	-	-	4	3	-	-	-	0.5
Senna (Coffee senna)	10	15	2	10	13	2	-	-	8	1	-	-	-	-	-	4.9
Sesbania (Hemp Sesbania)	-	-	1	-	-	8	5	1	-	-	-	-	-	1	-	1.1
Sida (Prickly sida)	1	1	1	5	11	6	7	12	5	8	-	-	-	-	-	3.8
Solanum (Hairy/Black/Silverleaf nightshades)	-	-	-	-	1	1	5	-	-	-	4	12	9	3	20	3.7
Xanthium Common Cocklebur	5	5	2	15	1	10	5	5	10	20	1	-	6	1	1	5.8

[1]Byrd Jr., 2003. Specific weed species not reported in Byrd Jr., 2003. Weed species added as representative of reported genus.

VIII.D.5. Methods of Weed Control in Conventional Cotton

From the early to mid-1900s weeds were removed from cotton using either hand hoeing or mechanical cultivation. It was not unusual for cotton to be cultivated weekly for the first 10 to 12 weeks of the growing season. As a result, weed control in a typical cotton production field at that time was best characterized as “one man, one mule and 10 to 20 acres of cotton” (Murray et al., 1992). In modern cotton production, a small but significant portion of U.S. cotton acreage continues to receive some type of ‘handweeding’ with 21% using hand labor annually in 1990 (Gianessi et al., 2002a, b). Today, hand hoeing is used primarily in California where 75% of its acres are estimated to use this method to some extent, with costs ranging from $15 to $150 per acre (Gianessi et al., 2002b; Vargas et al., 1996). Mechanical tillage to prepare weed-free seedbeds for planting and between row weed management continues in conventional cotton production systems with two to five cultivations used for weed control (Ridgway et al., 1984; Gianessi et al., 2002a, b).

The use of chemical methods for weed control began to develop in cotton in the 1940s and 1950s with the discovery and development of several selective herbicides. Beside herbicidal oils (various petroleum fractions), dinoseb, chloropropham, dalapon, monuron and diuron were developed and used in cotton. Cotton acreage treated with herbicides increased rapidly in the late 1950s with more than one million acres in the U.S. treated preemergence and 500,000 acres treated postemergence (McWhorter and Bryson, 1992).

Despite the increased use of herbicides in the late 1950s, less than 10 percent of the total U.S. cotton acreage received a herbicide treatment. However, herbicide use rapidly accelerated in the 1960s as a series of more selective herbicides were introduced. These herbicides provided good weed control with less cotton crop injury than most products used a decade earlier. These products included trifluralin, DSMA/MSMA, prometryn and fluometuron. By 1968, 91% of the U.S. cotton acreage was treated with at least one herbicide application (Bryson et al, 1999; Ridgway et al., 1984). Of these cotton herbicides developed in the 1950s and 1960s, trifluralin, MSMA, prometryn, fluometuron and diuron, representing varying chemical families and modes of action, are still widely used today.

During the 1970s, cotton producers began to develop management practices for the best weed control using tillage and combinations of the fairly effective herbicides that had been introduced in the 1960s. In addition, a number of new herbicides were introduced including five new dinitroanaline compounds (butralin, flurachloralin, dinitramine, profluralin and pendimethalin) and two additional triazines (cyanazine and dipropetryn). Because they were efficient, relatively economical and effective on a wide range of weed species, these herbicides continued to be used on U.S. cotton acreage with systems of two or more herbicides applied in combination at different cotton crop developmental stages. With the introduction of many novel and effective herbicides, almost all of the cotton acres received at least one herbicide application. Roundup agricultural herbicide (glyphosate), introduced in 1971, quickly became the most effective material for

nonselective ‘spot treatment’ of johnsongrass (*Sorghum halapense* L.) and other weeds (McWhorter and Bryson, 1992).

In the 1980s, a number of older herbicides including Dinoseb, were discontinued. Dinoseb had been widely used in cotton since its introduction in 1947 due to the fact that it was both highly effective and economical. In 1987, its usage for broadleaf weed control when applied in directed sprays was halted in with the suspension of the registration by the Environmental Protection Agency. Also discontinued were a number of herbicides introduced a decade earlier including dinitramine, flurachloralin, profluralin, dalapon, dipropetryn and perfluidone. During the 1980s a number of additional selective herbicide products including clomazone, sethoxydim, pyrithiobac and metolachlor were introduced to the cotton market for weed control although “the extent to which these would be used would not equal the acreage treated with the herbicides which were discontinued” (McWhorter and Bryson, 1992). As no single herbicide could replace the efficacy of dinoseb, combinations of other, more expensive herbicides, along with tillage, continued to be used into the 1990s (Bryson et al., 1999). A table of herbicides listing the expected levels of weed control is provided in Table VIII-8.

Table VIII-8. Expected Level of Control of Certain Grass, Broadleaf and Sedge Species.[1]

	Grass Weed Species[2]						Broadleaf Weed Species[2]							Sedges[2]		
Herbicides[3]	**BY**	**CG**	**GG**	**JGs**	**JGr**	**BG**	**CC**	**HS**	**MG**	**PW**	**PS**	**SP**	**DC**	**NSp**	**NSy**	**T**
Preplant																
Pendimethalin	9	9	9	9	5	0	0	0	3	9	0	1	0	0	0	G
Trifluralin	9	9	9	9	6	0	0	0	3	9	0	1	0	0	0	G
Preemergence																
Metolachlor	7	7	7	7	0	0	0	0	0	6	2	0	0	0	1	G
Diuron	7	8	8	6	0	0	4	4	7	9	6	5	0	0	0	G
Pyrithiobac	-	-	-	-	-	-	4	4	5	9	9	6	1	-	-	G
Post - OT[4]																
Fluazifop-P	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	E
Bromoxynil[7]	0	0	0	0	0	0	10	9	10	6	6	4	3	0	0	E
Glyphosate	9	9	8	9	8	6	8	6	8	9	9	8	8	8	7	G
Pyrithiobac	0	0	0	6	3	0	7	9	9	10	7	5	7	3	5	G
Post-Directed[5]																
Prometryn	7	7	7	7	-	6	8	7	-	-	-	1	6	-	G	
P + MSMA[6]	8	9	8	9	5	0	9	6	8	9	8	8	3	6	6	F
Fluometuron	6	6	6	6	-	5	6	5	-	-	-	-	0	4	-	G
F + MSMA	8	9	8	8	5	0	9	5	8	9	7	8	2	6	6	F
Diuron	5	6	5	5	2	0	4	4	7	7	4	8	3	0	0	G
D + MSMA	8	9	8	9	5	0	9	5	8	9	7	8	3	6	6	F
Glyphosate[7]	9	9	8	9	8	6	8	6	8	9	9	8	8	8	7	G

[1]Bryson et al., 1999 ; [2]Rating scale: 0-3, none to slight; 4-6, fair; 7-8, good; 9-10, excellent; [3]Applied according to label under optimum conditions; [4]OT = post emergent over the top application is a broadcast or banded application applies over the canopy of crops; [5] Postdirected = post emergent spray directed between the rows; [6]MSMA = monosodium methanearsonate, P, Prometryn, F, Fluometuron, D, Diuron; [7]Bromoxynil and glyphosate only used on BXN cotton or Roundup Ready cotton, respectively.
Weed Species: BY, barnyardgrass; CG, crabgrass; GG, goosegrass; JGs, johnsongrass (seedling); JGr, johnsongrass (rhizome); BG, bermudagrass; CC, common cocklebur; HS, hemp sesbania; MG, annual morningglory; PW, pigweed; PS, prickly sida; SP, sicklepod; DC, devil's claw; NSp, nutsedge (purple); NSy, nutsedge (yellow); T - Crop tolerance; rating scale: E, excellent; G, good; F, fair.

Estimated costs for full-season weed control in conventional cotton production vary based upon geography, production practices, and year. Gianessi reported that one-third of conventional U.S. cotton acreage received three or more herbicide applications in 1995, with two-thirds of the acreage receiving three or more cultivations for weed management (Gianessi et al., 2002a). Typically, cotton was treated with two to eight different herbicides applied over two to five applications. Two or sometimes three herbicides were tank-mixed and applied simultaneously. The average total cotton weed-control cost, including herbicides, application, tillage and handweeding, was estimated at $58.89 per acre (Gianessi et al., 2002a).

VIII.D.6. Methods of Weed Control in Herbicide-Tolerant Cotton

In 1995, the first herbicide tolerant cotton became available and provided tolerance to bromoxynil. During 1995, 50,000 acres of bromoxynil-tolerant (BXN) cotton were planted, and in 1996 about 2500 growers planted 200,000 acres of BXN cotton (Bryson et al., 1999). The second herbicide tolerant cotton product, Roundup Ready cotton, was introduced in 1997. Since its introduction, Roundup Ready cotton, used in combination with Roundup agricultural herbicides, has become the standard program for weed management in cotton. Glyphosate, the active ingredient in Roundup agricultural herbicides, provides broad-spectrum control of annual and perennial grass and broadleaf weeds. Roundup agricultural herbicides can be applied postemergence to Roundup Ready cotton from emergence through the four-leaf stage. However, after the four-leaf stage up to layby (canopy closure into the row), the herbicide must be applied as a post-directed spray between the crop rows to minimize contact with the cotton plant and prevent potential crop injury.

In 2002, Roundup Ready cotton was planted on approximately 59% of the cotton acres (USDA-NASS, 2003b). The primary advantage for growers using the Roundup Ready cotton system is the ease of postemergence herbicide application to control a broad spectrum of weeds with excellent crop safety (Wilcut et al., 2003). Additional benefits include simplicity and convenience, as well as a better fit into no-till and reduced tillage systems (Baldwin and Baldwin, 2002).

Research has not demonstrated that the Roundup Ready cotton system produces better weed control than that which can usually be obtained with conventional cotton and traditional herbicide systems. However, Roundup Ready cotton has expanded the grower's options for weed management and made the mechanics of weed control much easier, less expensive, and more convenient (Wilcut et al., 2003). Specifically, growers have reported making fewer trips across fields to apply herbicides and making fewer cultivation trips (Gianessi et al., 2002a).

Nonetheless, cotton growers continue to use a variety of herbicides with various modes of action in the production of Roundup Ready cotton (Table VIII-9). In 2001, glyphosate was the most widely used herbicide in cotton in terms of both the volume and area applied (USDA-NASS, 2002). However, the dinitroanaline herbicides, trifluralin and pendimethalin, were used on nearly half of the U.S. cotton acreage for small seeded

broadleaf and grass weed control. Further, various substituted urea herbicides (diuron, prometryn, fluometuron and linuron) were also used on 50% of the U.S. cotton acreage (USDA-NASS, 2002). Because of their soil residual activity on a number of weed species, these products can provide additional season-long control of continuously germinating weeds in the Roundup Ready cotton system (Wilcut et al., 2003; Askew et al., 2002). Other herbicide products representing additional modes of action, including carfentrazone, MSMA, pyrithiobac-sodium and metolachlor, were used on cotton acreages ranging from four to 11% (Table VIII-9). The values are comparable to other reported on a state-by-state basis (Byrd Jr., 2003).

Since herbicide tolerant cotton became available, USDA surveys of herbicide usage demonstrate a general decline in the overall amount of herbicide active ingredient used per acre for most states (Gianessi et al., 2002a). Cotton production savings of $8 to $20 per acre have been reported in the Mississippi Delta and savings of handweeding costs as high as $150 per acre have been reported for California (Gianessi et al., 2002a; Vargas et al., 1996). Overall, it has been estimated that cotton growers have experienced an annual reduction of $132 million in weed control costs because of the introduction of herbicide tolerant cotton (Gianessi et al., 2002a, b).

In a field study conducted at 22 locations in eight cotton-producing states, Wilcut et al., (2003) reported weed management costs (including herbicides and application, surfactant, seed, and technology fees, where appropriate) ranged from $74-76 per acre in conventional cotton compared to $49-72 per acre in a Roundup Ready cotton system. In a comparable study conducted in Tennessee, Hayes et al., (2001) reported that the highest returns were obtained with the glyphosate tolerant cultivars/glyphosate programs ($1300-1380 per hectare) and the conventional cultivar using a conventional herbicide program ($1330 per hectare). The conventional herbicide program included trifluralin incorporated as a preplant and fluometuron applied preemergence, followed by fluometuron plus MSMA post-directed and cyanazine plus MSMA post-directed at layby (Hayes et al., 2001). The complexity of the conventional herbicide system used as the standard treatment in this study illustrates the point that comparable yields can be obtained using a Roundup Ready cotton system with the additional benefit of increased convenience.

The adoption of the Roundup Ready cotton system has been found to encourage the adoption of conservation tillage practices. It has been estimated that for every two acres of Roundup Ready cotton and Bollgard/Roundup Ready cotton planted, one was also converted to reduced tillage (Kalaitzandonakes and Suntornpithug, 2001). A conservation tillage system can provide a range of economic, agronomic and environmental benefits including reduced fuel costs, reduced machinery investment, conservation of soil moisture, decreased soil compaction, decreased soil erosion from wind and water, better water infiltration, improved surface water quality, enhanced carbon sequestration and increased population and diversity of wildlife in and around fields (Baker and Laflen, 1979; Hebblethewaite, 1995; CTIC, 1998; Kay, 1995; CTIC, 2000; Phatak et al., 1999; Reicosky, 1995; Reicoski and Lindstrom, 1995).

Table VIII-9. Agricultural Chemical Applications Registered for Use in AK, GA, LA, MS, TX in 2001.[1]

Herbicide	Chemical Family	Mode of Action (MOA)	Area Applied (Percent)	Area Applied (Percent/MOA)	Total Applied (1000 lbs)	Total Applied (1000 lbs/MOA)
Glyphosate	Glycine	EPSPS inhibition	57	57	8,514	8,514
Trifluralin	dinitroanaline	Tubulin inhibitor	30	46	3,066	4,717
Pendimethalin	dinitroanaline	Tubulin inhibitor	16		1,651	
Diuron	substituted urea	PSII inhibitor	26	50	1,545	3,972
Prometryn	Triazine	PSII inhibitor	12		1,292	
Fluometuron	substituted urea	PSII inhibitor	10		977	
Linuron	substituted urea	PSII inhibitor	2		158	
Carfentrazone-ethyl	aryl triazinone	protox inhibitor	5	6	11	44
Lactofen	diphenylether	protox inhibitor	1		33	
MSMA	organic arsenical	cell membrane disruption	11	11	1,834	2,013
DSMA	organic arsenical	cell membrane disruption	< 1		179	
Pyrithiobac-sodium	benzoate	ALS inhibitor	10	10	85	85
S-Metolachlor	chloroacetamide	not well understood	4	4	419	419
Clethodim	cyclohexenone	ACCase inhibitor	2	2	28	28
Norflurazon	pyridazinone	carotenoid inhibitor	2	2	219	219
2,4-D (preplant)	phenoxy	auxin type	3	3	228	228

[1]Data derived from USDA-NASS, Agricultural Statistics Board. Agricultural Chemical Usage 2001 Field Crops Summary (USDA-NASS, 2002). Bromoxynil (applied area of 1%) which is only used with BXN cotton, and cyanazine (use discontinued) were not transferred from USDA-NASS table.

VIII.D.7. Roundup Ready Flex Cotton MON 88913

MON 88913 will offer cotton farmers an improved product for management of economically damaging weeds. As noted in the previous section, over-the-top applications of a Roundup agricultural herbicide to Roundup Ready cotton can be made from crop emergence through the fourth leaf (node) stage of development. Because of the potential for boll loss, delayed maturity and yield loss, applications from the fifth leaf stage through layby must currently be post-directed under the crop canopy in order to minimize foliar contact. Sequential over-the-top or post-directed applications of a Roundup agricultural herbicide must be at least ten days apart and cotton must have at least two nodes of incremental growth between applications.

Because of increased tolerance to glyphosate in reproductive tissues, MON 88913 demonstrates an increased margin of fruit retention and crop safety. This will allow for an expanded window of over-the-top applications of a Roundup agricultural herbicide, extending from cotton emergence through layby. Weed control at these early and mid-stages of cotton growth is critical to eliminate the potential for weeds to compete for limited water, sunlight and plant nutrients. The grower will be able to more effectively manage his/her weed control in cotton using over-the-top herbicide applications when compared to post-directed or hooded-sprayer applications. Directed herbicide application requires specialized equipment that is often susceptible to misapplication, must be operated at lower speeds and requires a greater number of trips per acre compared to larger broadcast applicators. Additional anticipated benefits of using MON 88913 include increases in cropping efficiency by combining, in a single application, a Roundup agricultural herbicide and the other crop chemical products. For example, various foliar insecticides may be combined with Roundup agricultural herbicides during the season for secondary pests such as thrips, aphids, and plant bugs, depending on economic thresholds for treatment. Additionally, mepiquat chloride, a plant growth regulator commonly used in cotton production to reduce vegetative growth and increase fruit retention, may be applied. The anticipated timing of Roundup agricultural herbicide applications in MON 88913 relative to growth stages of Roundup Ready cotton is presented in Table VIII-10.

Table VIII-10. Anticipated Weed Control Options/Herbicide Use in MON 88913 Compared to the Current Roundup Ready Cotton Product.[1]

Cotton Growth Stage	Roundup Ready Cotton	MON 88913[2]
PrePlant burndown	*Over- the-top:* Roundup herbicide[3] Contact herbicides[4] Residual herbicides[4]	*Over-the-top:* Roundup herbicide Contact herbicides Residual herbicides
PrePlant	Residual herbicides/ Tillage	Residual herbicides/ Tillage
Cracking through 4th node	*Over-the-top:* Roundup herbicide / Tillage	*Over-the-top:* Roundup herbicide / Tillage
4th node through layby	*Post-directed/hooded sprayers:* Roundup herbicide Other in-crop herbicides[4]	*Over-the-top:* Roundup herbicide Other in-crop herbicides *Post-Directed:* Other in-crop herbicides
After layby	Roundup herbicide[5] (salvage treatment only)	*Over-the-top:* Roundup herbicide
Preharvest interval	Up to 7 days	Up to 14 days

[1] Total Roundup herbicide application limited to 6.0 lb. glyphosate acid per acre per year.
[2]New rates and timings for MON 88913 subject to Roundup agricultural herbicide label registration.
[3]Any of the Roundup family of agricultural herbicides.
[4]Non-glyphosate-based herbicides with other modes of herbicidal activity that are labeled for use in cotton.
[5]Salvage treatment will result in fruit loss and resulting yield loss.

VIII.D.8. Volunteer Management

VIII.D.8.a. Crop Rotational Practices in Cotton

In general, the appropriate use of crop rotation can reduce disease, nematode, and insect populations, and increase organic matter and soil fertility. For these reasons, rotational cropping in some form dominates most U.S. major crop production. According to USDA Economic Research Service (ERS) data based upon Agricultural Resource Management Study (ARMS) surveys, in their primary states of production, ≥ 98% of peanut, sunflower and potato acreage is cultivated in a crop rotation. Soybean and corn are only slightly lower at 92% and 84%, respectively (USDA-ERS, 2003a). Cotton, however, is an exception to this, with only 39% of cotton acreage in 1999 grown in a rotation with other row crops and small grains (USDA-ERS, 2003a). The remaining 61% was grown as continuous cotton.

Due to the continued adoption of Roundup Ready cotton, soybean and corn, the potential for occurrence of Roundup Ready cotton volunteers has been assessed. Table VIII-11 summarizes the cropping patterns for the major cotton producing states from 1996 to 2000. These data were collected either from a recent publication by Gianessi and Sankula (2003), or by the USDA-ERS through its ARMS surveys. In the case of Roundup Ready soybean, the percentage and acreage would be expected to be equivalent to the herbicide tolerant values provided (e.g. there are no other commercial herbicide tolerant soybean products available). Values for herbicide-tolerant cotton and corn would include minimal acreages for BXN cotton and Liberty Link® corn, respectively. The vast majority of cotton grown during this five-year period was in a continuous cropping system (73.9%), with only 26.1% of the acreage rotated to other crops on average (Table VIII-11). Cotton is produced in rotation with corn, soybeans and other crops (other row crops, small grains and fallow or idle land) in varying amounts in each state (Table VIII-11). Corn is a relatively minor rotational crop in most states, with Louisiana and South Carolina utilizing the largest acreage in rotation. Similarly, soybean is a relatively minor rotational crop in most states with exception of South Carolina. As reported by Bryson et al., cotton is rotated to an even lesser extent with milo (*Sorghum vulgare* Pers.), peanuts (*Arachis hypogaea*), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) depending upon geography (Bryson et al., 1999; USDA-ERS, 2003a).

In summary, crop rotation in cotton production varies according to state and local practices, but on the average over the past several years, approximately 74% of acres are not rotated in the subsequent season to another crop. Despite potential advantages in using a crop rotation to enhance options in weed control, reduce diseases and pests, and enhance soil fertility, many growers grow continuous cotton. The decision not to rotate to another crop is primarily financial. Other factors may include increased field management requirements because of rotational crop herbicide restrictions and the knowledge that some cover crops are detrimental to cotton production (Bryson et al., 1999). The ability to manage weeds, pests and diseases weigh heavily into a farmer's

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decision to employ a crop rotation. As discussed above, weed management in cotton employs a wide variety of effective tools, from tillage to utilization of a wide selection of registered herbicides employing different modes of action, and weed management systems using biotechnology-derived cotton. Thus, for weed control in cotton, crop rotation is often not the primary option employed by the majority of farmers.

VIII.D.8.b. Cotton Volunteer Management

Volunteer cotton refers to plants that have germinated, emerged and established unintentionally. Volunteers generally come from seed that falls to the ground (often because of late season storms) in the previous cotton crop and overwinters. Occurrence of volunteers depends on tillage after harvest and the severity of winters. Cultivation and herbicides have traditionally been the most common methods of volunteer cotton control. Both require that the cotton plants are germinated and have emerged before control can occur. If the volunteer cotton plants contain the Roundup Ready trait, then the use of glyphosate alone in subsequent crops will not control these seedlings. Thus, alternative mechanical or herbicide measures may be required (Roberts et al., 2002) and are widely available.

Because of the ongoing planting of continuous cotton and the large acreage of Roundup Ready cotton currently planted, the majority of MON 88913 volunteers would be expected to occur in the subsequent MON 88913 crop. Control of these volunteers would follow the preplant and in-crop mechanical and chemical methods currently used for volunteer Roundup Ready cotton as discussed above. In other Roundup Ready crops in the rotation, MON 88913 volunteers would be expected less frequently in Roundup Ready soybean and corn because of the lack of significant rotation of cotton to these crops (Table VIII-11), combined with the level of herbicide tolerant trait adoption. As a result, the Roundup Ready corn and soybean acreage on which volunteer MON 88913 may be expected to occur is relatively small. The herbicide control options available in corn and soybean will continue to result in the ability to manage cotton volunteers.

In the U.S., volunteer cotton including Roundup Ready cotton has been infrequently encountered emerging in rotational crop fields in the cottonbelt. Cotton volunteers, when they occur, usually emerge in conservation tillage systems where tillage is not used for vegetation control prior to planting or after emergence of the crop. University researchers from a number of cotton-producing states have recommended effective and economical control of cotton volunteers by mechanical tillage and alternative herbicides (Roberts et al., 2002).

Mechanical tillage prior to planting is an effective and efficient method for controlling seedling volunteer cotton plants, including Roundup Ready cotton volunteers. This is accomplished in most soil conditions because the root and hypocotyls of seedling cotton are easily destroyed by the cultivation process. Any damage occurring below the cotyledons will kill the plant because there are no growing points from which the plant can recover (Roberts et al., 2002). Cultivation will also manage other weeds (Alford et al., 2002; Murdock et al., 2002) (Tables VIII-12A, B). The disadvantages of cultivation are moisture loss under arid conditions and the possibility of increased soil erosion.

Alternatively, the use of a non-glyphosate herbicide (such as paraquat, dicamba or flumioxazin) as a preplant burndown treatment prior to planting will eliminate emerged volunteer cotton (Murdock et al., 2002; Montgomery et al., 2002; Roberts et al., 2002). In most situations, these preplant measures are sufficient, and the need for additional control measures specifically for cotton volunteers is not required. In those rare cases where further control measures are required, these preplant steps generally increase the effectiveness of later in-crop weed and cotton seedling volunteer control measures in cotton, corn, soybean, and other crops.

In emerged cotton, labeled non-glyphosate herbicides such as carfentrazone or paraquat may be applied through hooded sprayers or other selective equipment to effectively control volunteer plants and other weeds in row middles (Alford et al., 2002; Murdock et al., 2002; Montgomery et al., 2002). Special care must be taken, however, to ensure that the nonselective herbicide does not contact the cotton crop (Gray et al., 2002). A number of herbicides provide control of Roundup Ready cotton volunteers in soybean (York et al., 2002; Clemmer et al., 2001), including chlorimuron and imazaquin (Tables VIII-12A, B). Volunteer cotton in a rotation to corn generally is not a problem because of the sensitivity of cotton to a number of commonly used corn herbicides (e.g., atrazine).

In emerged cotton, mechanical tillage in the form of a standard cultivator has been traditionally used in the subsequent cotton crop to effectively remove weeds and volunteer cotton plants between the crop rows. In reduced tillage situations, high residue cultivators with sweeps may be used to effectively lift weeds out of the soil to leave the ground cover undisturbed. Cotton emerged within the row can negatively impact cotton growth and management decisions due to increased plant population and disease susceptibility (Roberts et al., 2002). However, plants remaining at the end of the season can generally be harvested with the planted population by mechanical picking or stripping.

Table VIII-11. Estimated U.S. Cotton and Crop Rotation, Roundup Ready Crop Percentages and Roundup Ready Crop Rotation Percentages by State.

									Estimated Roundup Ready Crop in rotation with Roundup Ready Cotton[5]		
State	Cotton Acreage[1]	Cotton/ Cotton Rotation[2]	Roundup Ready Cotton Acreage[3]	Corn/ Cotton Rotation[2]	Roundup Ready Corn Acreage[3]	Soybean/ Cotton Rotation[2]	Roundup Ready Soybean Acreage[3]	Other Crops[4]/ Cotton Rotation[2]	Roundup Ready Cotton	Roundup Ready Corn	Roundup Ready Soybean
		Percent									
AL	560,000	68.5	70.0	4.1	15.0	3.7	76.0	23.8	48.0	0.6	2.8
AR	950,000	93.3	71.0	3.9	15.0	4.3	84.0	3.0	66.2	0.6	3.6
AZ	204,000	67.7	70.0	*	15.0	*	76.0	33.5	47.4	na	na
CA	700,000	67.3	30.0	*	15.0	*	76.0	27.8	20.2	na	na
GA	1,400,000	68.3	79.0	5.0	15.0	2.8	76.0	24.0	54.0	0.8	2.1
LA	550,000	77.9	61.0	17.4	15.0	4.8	76.0	4.4	47.5	2.6	3.7
MO	400,000	86.4	70.0	9.6	10.0	*	83.0	*	60.5	1.0	na
MS	1,120,000	90.2	77.0	9.3	15.0	5.9	89.0	2.6	69.5	1.4	5.3
NC	850,000	61.3	77.0	6.0	15.0	14.1	76.0	18.7	47.2	0.9	10.7
OK	190,000	*	70.0	*	15.0	*	76.0	*	na	na	na

[1]USDA-NASS, 2003b.

[2]Estimates based upon cotton and crop rotation (corn, soybean, other crops) percentages provided by USDA Economic Research Services (ERS) to Monsanto, August 2003. Information collected by USDA-ERS in Agricultural Resource Management Study (ARMS) surveys 1996-2000. Some states not reported in all years.

[3]Percentage of total area planted, USDA-NASS, 2003b, p. 24 (corn), p. 25 (cotton), and p. 25 (soybean). State percentages used when provided, national average was used if state percentage was not provided. Average percent of Roundup Ready corn acreage estimated from total percentage of all herbicide tolerant and stacked gene varieties.

[4]Other rotational crops may include other row crops, small grains, meadow, fallow or idle as collected in USDA-ERS ARMS surveys.

[5]Based on U.S. average of continuous cotton (e.g., non-rotated) or rotational crop percentage values multiplied by respective herbicide tolerant crop percentages.

*Values not provided by USDA-ERS for reasons of confidentiality; na: data not available.

Table VIII-11 (Continued). Estimated U.S. Cotton and Crop Rotation, Roundup Ready Crop Percentages and Roundup Ready Crop Rotation Percentages by State.

									Estimated Roundup Ready Crop in rotation with Roundup Ready Cotton[5]		
State	Cotton Acreage[1]	Cotton/ Cotton Rotation[2]	Roundup Ready Cotton Acreage[3]	Corn/ Cotton Rotation[2]	Roundup Ready Corn Acreage[3]	Soybean/Cotton Rotation[2]	Roundup Ready Soybean Acreage[3]	Other Crops[4]/ Cotton Rotation[2]	Roundup Ready Cotton	Roundup Ready Corn	Roundup Ready Soybean
		Percent									
SC	250,000	55.7	70.0	11.4	15.0	26.6	76.0	6.2	39.0	1.7	20.2
TN	560,000	87.7	70.0	4.3	15.0	11.6	76.0	1.7	61.4	0.7	8.8
TX	5,816,000	69.7	45.0	7.5	15.0	0.7	76.0	22.6	31.4	1.1	0.5
U.S.	**13,924,000**	**73.9%**	**59.0%**	**6.3%**	**15.0%**	**2.4%**	**81.0%**	**17.4%**	**43.6%**	**0.9%**	**1.9%**

Table VIII-12A. Effectiveness of Tillage and Alternative Herbicides for the Control of Roundup Ready Cotton in a Continuous Cotton or Cotton/Soybean Crop Rotation.

	Tennessee	South Carolina	Mississippi	Louisiana
Previous Crop	Roundup Ready cotton	Roundup Ready cotton	Roundup Ready cotton	Roundup Ready cotton
Rotational Crop	Roundup Ready cotton	Roundup Ready cotton	Roundup Ready cotton	Roundup Ready cotton
Control Method/ Level of Control				
Tillage/Cultivation	>95%	100%	(not tested)	(not tested)
Alternative Herbicides*	**52 to >95% (in-crop)** Gramoxone® (paraquat) Direx® (diuron) Liberty® (glufosinate) Valor® (flumioxazin) Aim™ (carfentrazone) Clarity® (dicamba)	**92-100% (preplant)** Gramoxone (paraquat) Valor (flumioxazin) Weedone® LV4 (2,4-D) Clarity (dicamba) Cobra® (lactofen)	**90%** diuron/cultivation	**82-87% (preplant)** Canopy® (chlorimuron) Canopy XL® (sulfentrazone + chlorimuron) Python® (flumetsulam) **80% (in-crop)** 2,4-DB (2,4-DB)
Researchers	Alford et al., 2002	Murdock et al., 2002	Gray et al., 2002	York et al., 2002
University	U. of Tennessee	Clemson	Mississippi State	Louisiana State

*Brand name (common name). Applied preplant broadcast or in-crop using a hooded sprayer.

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Table VIII-12B. Effectiveness of Tillage and Alternative Herbicides for the Control of Roundup Ready Cotton/Soybean in a Cotton Crop Rotation.

	North Carolina	North Carolina/Georgia	Australia
Previous Crop	Roundup Ready cotton	Roundup Ready cotton	Roundup Ready cotton
Rotational Crop	Roundup Ready soybean	Roundup Ready soybean	*[2]
Control Method/ Level of Control			
Tillage/Cultivation	(not tested)	(not tested)	(not tested)
Alternative Herbicides[1]	**62-93% (preplant)** Sencor® (metribuzin) Canopy XL (sulfentrazone + chlorimuron Scepter® (imazaquin) Python™ (flumetsulam) **71-84% (in-crop)** Classic® (chlorimuron) Reflex® (fomesafen) 2,4-DB (2,4-DB) Resource® (flumiclorac)	**72-97% (preplant)** Canopy XL (sulfentrazone + chlorimuron Canopy (chlorimuron) Python (flumetsulam) Scepter (imazaquin) **83-99% (in-crop)** Reflex (fomesafen) Classic (chlorimuron) Resource flumiclorac)	**100% (preplant)** Bromoxynil Carfentrazone Paraquat + Diquat Glufosinate-ammonium (92% preplant)
Researchers	York et al., 2002	Clemmer et al., 2001	Roberts et al., 2002
University/Organization	North Carolina State	North Carolina State	CSIRO, QDPI & NSW Agric.[3]

[1]Brand name/common name. Applied preplant or in-crop using a hooded sprayer.
[2]Not examined.
[3]CSIRO, Commonwealth Scientific and Industrial Research Organisation; QDPI, Queensland Department of Primary Industries; NSW Agric., New South Wales Agriculture (Minister for Agriculture and Fisheries).
® Sencor is a registered trademark of Bayer; ® Scepter is a registered trademark of BASF Agrochemical Products B.V.; ™ Python is a trademark of Dow Agrosciences LLC; ® Classic is a registered trademark of E. I. du Pont de Nemours and Company; ® Reflex is a registered trademark of Syngenta Crop Protection Inc.; ® Resource is a registered trademark of Valent U.S.A. Corporation.

In summary, cotton farmers have a wide of range of options for controlling weeds and volunteers. These options include tillage and residual and contact herbicides. Roundup Ready cotton has expanded the grower options for weed management and made the mechanics of weed control much easier, cost effective, and more convenient. Despite the significant adoption of Roundup Ready cotton (approximately 59% of U.S. cotton acres), cotton growers continue to use a variety of herbicides with various modes of action in cotton. In 2001, glyphosate was the most widely used herbicide in cotton (volume and area applied); however, triflualin and pendimethalin herbicides were used on nearly half of the U.S. cotton acres, and substituted-urea herbicides (diuron, prometryn, and linuron) were used on 50% of the U.S. cotton acreage. Because of their soil residual activity, these products can provide additional season-long control of continuously germinating weeds in the Roundup Ready cotton system.

As discussed in Sections VI., VII. and VIII., regarding the nature and safety of the CP4 EPSPS protein, the phenotypic comparisons between MON 88913 and MON 88913(-), and the current volunteer control methods in cotton production, there is no reason to believe that the incidence of MON 88913 volunteers in the subsequent crop (either cotton or another rotational crop) would be expected to be any different than for the current Roundup Ready cotton product. The incidence of MON 88913 volunteers in the subsequent crop would be expected to be low due to (1) the limited use of crop rotations in cotton production; (2) cotton is only capable of producing volunteers in regions where freezing conditions do not occur, and is thus not able to volunteer in much of the continental U.S. Therefore, MON 88913 volunteers would not be expected to generate volunteer crop problems for the grower.

VIII.E. Weed Resistance to Glyphosate

The risk of weeds developing resistance and the potential impact of resistance on the usefulness of an herbicide vary greatly across different modes of action and are dependent on a combination of different factors. Monsanto considers product stewardship to be a fundamental component of customer service and business practices and invests considerably in research to understand the proper uses and stewardship of the glyphosate molecule. This research includes an evaluation of some of the factors that can contribute to the development of weed resistance. Further information regarding glyphosate stewardship is presented in Appendix F.

IX. Summary of Environmental Assessment

The phenotypic evaluations of MON 88913 included an assessment of seed dormancy, germination and emergence, vegetative growth, reproductive growth, seed retention on the plant, seed compositional analyses, and reproductive comparisons and environmental interactions to field stressors. These studies were conducted across a broad range of environmental conditions and agronomic practices to represent the conditions that MON 88913 would likely encounter in commercial production. These detailed characterizations and comparisons demonstrate that, with the exception of production of the CP4 EPSPS protein, MON 88913 poses no greater pest potential than conventional cotton currently grown in the U.S.

The environmental consequences of pollen transfer from MON 88913 to other cotton or other related *Gossypium* species is considered to be negligible because of limited movement of cotton pollen, the safety of the introduced protein, and the lack of any selective advantage that might be conferred on the recipient feral cotton or wild relatives. Additionally, the potential for outcrossing to sexually compatible species is also unlikely because of the lack of significant populations of sexually compatible related species of cotton existing in the principle regions of cotton production in the U.S. The agronomic consequences of volunteer MON 88913 cotton plants are expected to be minimal as these plants are easily controlled by mechanical means or by one of a number of herbicides currently registered for control of cotton. There is no indication that MON 88913 would have an adverse impact on organisms beneficial to plants or to non-pest organisms, including threatened or endangered organisms.

From an ecological perspective, MON 88913 is similar to the commercial Roundup Ready cotton product used in the U.S. since 1997. MON 88913 is expected to rapidly replace the majority of Roundup Ready cotton acres. Farmers familiar with the Roundup Ready cotton system would continue to employ the same crop rotational practices and/or volunteer control measures currently in place for Roundup Ready cotton. MON 88913 provides enhanced reproductive tolerance relative to Roundup Ready cotton and will therefore provide a wider window for over-the-top glyphosate-based herbicide applications.

X. Adverse Consequences of Introduction

Monsanto knows of no study results or observations associated with Roundup Ready Flex cotton MON 88913 that would be anticipated to result in adverse environmental consequences from introduction. MON 88913 is a second-generation biotechnology cotton product that expresses the Roundup Ready trait. As demonstrated by field results and laboratory tests, the only biologically relevant phenotypic difference between MON 88913 and conventional cotton is the CP4 EPSPS protein produced in MON 88913 that provides tolerance to Roundup agricultural herbicides. This protein is identical to that found in the current Roundup Ready cotton product, which is grown on a majority of U.S. cotton acres and comprises a significant portion of the annual U.S. cotton crop.

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Appendix A: USDA Notifications

Appendix A: USDA Notifications

Field trials of Roundup Ready Flex cotton, MON 88913, were conducted in the U.S. beginning in 2000. The protocols for these trials included assessments of crop tolerance to Roundup agricultural herbicides, field performance, agronomics, and the generation of field materials and data necessary for this petition. In addition to the phenotypic assessment data provided for MON 88913, observational data on pest and disease stressors were collected from these product development trials. Most of these final reports have been submitted to the USDA. Final reports for the 2003 field trials are still in preparation and will be submitted when completed. These observational data provide confirmatory evidence to support the quantitative phenotypic characterization data and assessment provided in Section VII. A list of trials conducted under USDA notification is presented in Table A-1. Final reports that have not yet been submitted to the USDA are noted with an asterisk.

Table A-1. MON 88913 Field Trial Notification Numbers.

USDA Reference Number	Effective Date	Approved Release Sites (by state) Covered by Notification
2000 Field Trials:		
00-038-23n	3/9/00	PR
00-042-02n	3/12/00	PR
00-059-06n	3/29/00	PR
00-089-13n	5/3/00	MS
00-118-10n	5/19/00	GA
00-140-06n	6/22/00	PR
00-213-01n	9/11/00	PR
2001 Field Trials:		
00-362-01n	1/29/01	AZ, TX
01-031-02n	3/22/01	AL, AR, AZ, GA, MS, NC, SC, TN, TX
01-058-07n	3/29/01	IL
01-232-02n	9/20/01	PR
2002 Field Trials:		
02-004-11n	2/3/02	TX
02-016-27n	2/15/02	LA
02-018-16n	2/17/02	AZ
02-022-50n	2/21/02	CA
02-022-54n	3/26/02	AL
02-022-55n	2/21/02	MO
02-023-15n	3/20/02	TN
02-023-16n	2/27/02	AL, AR, LA, MS, NC, SC, TX
02-025-01n	2/24/02	NC, SC
02-025-02n	2/24/02	MS
02-025-07n	2/24/02	GA
02-025-08n	2/24/02	TX
02-025-09n	2/24/02	IL
02-028-28n	2/27/02	AR
02-042-31n	3/13/02	AL, CA, GA, TX
02-044-12n	3/15/02	AR, AZ, GA
02-046-12n	3/17/02	AR, GA, MS, OK
02-046-14n	3/17/02	TX
02-046-15n	3/17/02	AZ
02-051-22n	3/22/02	CA
02-221-08n	9/11/02	PR

Table A-1 (Continued). MON 88913 Field Trials.

USDA Reference Number	Effective Date	Approved Release Sites (by state) Covered by Notification
2003 Field Trials:		
02-282-09n*	11/21/02	AZ, MS, TX
03-022-06n*	2/21/03	TX
03-023-03n*	2/22/03	TN
03-027-01n*	2/26/03	AL, GA, MS, NC
03-027-03n*	2/26/03	TN
03-030-05n*	3/31/03	AL, AR, AZ, FL, GA, LA, MO, MS, NC, OK, SC, TX
03-030-12n*	3/1/03	CA
03-038-02n*	3/9/03	AZ, MS, TN
03-042-10n*	3/13/03	AZ
03-042-11n*	3/13/03	AL
03-042-12n*	4/4/03	GA
03-042-13n*	3/13/03	MS
03-042-14n*	3/13/03	TX
03-042-19n*	3/13/03	AZ, CA
03-043-13n*	3/14/03	OK
03-052-23n*	3/23/03	TX
03-052-29n*	3/23/03	AR, CA, MS
03-052-45n*	3/23/03	TX
03-052-46n*	3/23/03	TX
03-052-47n*	3/23/03	AZ, MS
03-059-03n*	3/30/03	MS, SC
03-071-04n*	4/11/03	AR
03-100-03n*	5/10/03	IL
03-112-11n*	5/22/03	GA
03-115-04n*	5/25/03	AR
03-224-02n*	9/11/03	PR
03-226-04n*	9/23/03	PR
03-226-05n*	9/23/03	PR
03-226-06n*	9/23/03	PR
03-226-07n*	9/13/03	PR
03-226-08n*	9/13/03	PR
03-226-09n*	9/13/03	PR
03-226-10n*	9/13/03	PR
03-227-01n*	9/23/03	PR
03-227-02n*	9/14/03	PR
03-317-01n*	12/13/03	AR, TX

*Final reports in preparation

Appendix B: Materials and Methods

B.1. Molecular Characterization

B.2. Protein Characterization

B.3. Protein Levels

B.4. Phenotypic Evaluation

Appendix B: Materials and Methods

B.1. Molecular Characterization

Materials

DNA for the analysis was isolated from MON 88913 and MON 88913(-) cottonseed produced under field conditions in 2002 (Appendix C, Section C.3.). Additional DNA extracted from cottonseed from MON 88913 breeding generations was used in generational stability analyses. For these analyses, DNA was isolated from leaves or seed. The control was MON 88913(-). MON 88913(-) is a negative segregant derived from MON 88913 that does not contain the DNA insert. The references included the plasmid PV-GHGT35 (Figures V-1a and V-1b) that was used to produce MON 88913. For Southern blot analyses of cotton genomic DNA, digested DNA of plasmid PV-GHGT35 (~0.5 and 1 genome copy equivalents) was mixed with digested DNA from MON 88913(-) and separated by electrophoresis on agarose gels. As additional reference standards, the 1 kb DNA Extension Ladder from Invitrogen was used for size estimations on Southern blots. The High Mass Ladder and 1 kb Ladder from Invitrogen were used for size estimations for the PCR analyses.

Characterization of the Materials

The identity of the field-produced cottonseed was confirmed by PCR analysis prior to use to confirm the presence or absence of MON 88913, as appropriate. The stability was determined in each Southern analysis by observation of the digested DNA sample on an ethidium bromide stained agarose gel. The identity of the materials used in generational stability analyses was confirmed by chain-of-custody documents and by Southern blot fingerprint.

DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA from MON 88913 and MON 88913(-) was extracted from cottonseed by first grinding the seed to a meal and then following standard DNA extraction procedures based on the CTAB DNA extraction method of Rogers and Bendich (1985). Genomic DNA samples were incubated in a 65°C water bath prior to quantification (typically for several hours). Leaf tissue used in the stability analyses was lyophilized for ~23 hours and then ground into a fine powder. The genomic DNA was extracted following standard procedures. Genomic DNA was stored in a 4°C refrigerator. The DNA from plasmid PV-GHGT35 was purified from a ~50 ml culture of LB broth inoculated with PV-GTGT35 from an *E. coli* glycerol stock. The culture contained ~50 µg/ml spectinomycin and was grown in a 37°C shaking incubator overnight. The DNA was isolated from the *E. coli* using a Qiagen Midi extraction kit (Catalog # 12243) as per the manufacturer's instructions. Purified plasmid DNA was stored in a -20°C freezer.

Quantification of Genomic DNA

Quantification of DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer with Roche Molecular Size Marker IX or Roche pBR322 DNA as a calibration standard.

Restriction Enzyme Digestion of Genomic DNA

Approximately 20 µg of genomic DNA from either MON 88913 or MON 88913(-) were used for restriction enzyme digestions. Overnight digests were performed at 37°C according to standard procedures based on Sambrook and Russell (2001) in a total volume of 500 µl using 100 units of the appropriate restriction enzyme(s). After digestion, the samples were precipitated by adding 1/10 volume (50 µl) of 3 M NaOAc (pH 5.2) and two volumes (1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer. The digested DNA was precipitated at maximum speed in a microcentrifuge, washed with 70% (v/v) ethanol, vacuum-dried, and re-dissolved in TE buffer.

DNA Probe Preparation for Southern Blot Analyses

Probe template DNA containing sequences of plasmid PV-GHGT35 (Figures V-1a and V-1b) was prepared by PCR amplification following a standard procedure based on Sambrook and Russell (2001). Approximately 25 ng of each probe template were labeled with ^{32}P-dCTP (~6000 Ci/mmol) at 65°C or with ^{32}P-dATP (~6000 Ci/mmol) at 60°C (T-E9 and P-FMV/TSF1+ L-TSF1/I-TSF1 probes) by the random priming method (RadPrime DNA Labeling System, Life Technologies).

Southern Blot Analyses of Genomic DNA

Samples of DNA digested with restriction enzymes were separated based on size using 0.8% (w/v) agarose gel electrophoresis according to standard procedure based on Sambrook and Russell (2001). A 'long run' and 'short run' were performed during the gel electrophoresis. The ~20 µg samples of digested MON 88913 DNA were divided in half for loading ~10 µg on the long run and ~10 µg on the short run. The long run enabled greater separation of higher molecular weight DNAs, while the short run allowed smaller molecular weight DNAs to be retained on the gel. The long-run samples were loaded onto the gel and typically subjected to electrophoresis for 14-16 hours at 35 volts. The short run samples were then loaded in adjacent lanes on the same gel, and typically the gel was subjected to electrophoresis for 4–5 additional hours at 85 volts. In the case of generational stability analyses, ~10 µg of digested genomic DNA were separated based on size using a 0.8% (w/v) agarose gel as a single run at 35 volts for ~18.5 hours. All Southern blot analyses were performed according to standard procedure based on the method of Southern (1975). Multiple exposures of each blot were then generated using Kodak Biomax MS-1 or MS-2 film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

PCR Analyses of the Insert

The organization of the elements within the DNA insert and verification of adjacent genomic cotton DNA in MON 88913 were confirmed using PCR analysis by amplifying six overlapping regions of DNA that span the entire length of the insert. The PCR

analyses were conducted using 50 ng of genomic DNA template in a 50 µl reaction volume containing a final concentration of 1.5 mM $MgCl_2$, 0.2-1.22 µM of each primer, 0.2 mM each dNTP, and 2.5 µl of HotStarTaq DNA polymerase (Qiagen). The amplification of Products A-F was performed under the following cycling conditions: 95°C for 15 minutes, 38 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, and 1 cycle at 72°C for 2 minutes. Aliquots of each product were separated on 1.0 % (w/v) agarose gels and visualized by ethidium bromide staining to verify the products were of the expected size.

B.2. Protein Characterization

An assessment of the equivalence of the MON 88913-produced protein to a previously characterized *E. coli*-produced CP4 EPSPS protein was conducted. This characterization was conducted in accordance with Monsanto's standard procedures.

Materials

The plant-produced CP4 EPSPS protein was isolated from seed of MON 88913 produced under field conditions in 2002 (Appendix C, Section C.3.). The identity of the seed was confirmed by PCR analysis. The CP4 EPSPS protein was stored in a −80°C freezer in a buffer solution containing 50 mM Tris-Cl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine, and 25% (v/v) glycerol at a total protein concentration of 0.5 mg/mL.

Description of the CP4 EPSPS Protein Reference Standard

E. coli-produced CP4 EPSPS protein (Monsanto Analytical Protein Standard lot 20-100015) was used as a reference standard to establish equivalence in select analyses. These analyses included molecular weight determination by SDS-PAGE, immunoblot analysis and the functional enzymatic assay.

Controls

Protein molecular weight standards were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF membranes. β-Lactoglobulin protein and PTH-amino acid standards were used to verify the performance of the amino acid sequencer. The following standards and controls were used during amino acid analysis: NIST BSA, NIST AA standards, and norvaline standard.

Protein Purification and Confirmation

The CP4 EPSPS protein was purified from extracts of ground MON 88913 seed using a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography, anion exchange chromatography, and affinity chromatography. The identity of the plant-produced CP4 EPSPS protein was confirmed using two analytical methods, N-terminal sequencing and immunoblotting. The total protein concentration of the plant-produced CP4 EPSPS protein sample was estimated to be 0.5 mg/mL using amino acid analysis.

N-terminal Sequence Analysis

Prior to N-terminal sequence analysis, five 5 µg aliquots of the plant-produced CP4 EPSPS protein in Laemmli sample buffer were electrophoresed and then electrotransferred to a 0.2 µm PVDF membrane (Bio-Rad). Prior to electrophoresis, the samples were first heated to ~100°C for 5 min and cooled. These samples, along with pre-stained molecular weight markers (Bio-Rad Dual Color, cat # 161-0374, Hercules, CA), were loaded onto a pre-cast Tris-Glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 140 V for 18 min followed by a constant voltage of 200 V for 52 min until the dye front approached the bottom of the gel. The gel was then electroblotted for 60 min at a constant current of 300 mA in a solution containing 10 mM CAPS diluted with 10% (v/v) methanol, pH 11. Protein bands were stained by soaking the membrane for 90 sec in Ponceau S stain (Sigma, St. Louis, MO) and destained by washing twice with Milli Q water each for 2 minutes. Two lanes of the CP4 EPSPS protein band at ~43 kDa were excised from the membrane and sequenced.

N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785A Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas^{99} software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the ~43 kDa protein band to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

Immunoblot Analysis – Immunoreactivity

Aliquots of the stock solutions of the plant-produced CP4 EPSPS and reference standard were diluted in Laemmli sample buffer (Laemmli, 1970) to final concentrations of 0.3, 0.2, and 0.1 ng/µL. Samples were then heated to 97°C for five min and applied to a pre-cast Tris-Glycine 4→20% polyacrylamide gradient 15-well mini-gel (Invitrogen, Carlsbad, CA). Both plant- and *E. coli*-produced CP4 EPSPS proteins were loaded in duplicate at 1, 2, and 3 ng CP4 EPSPS protein per lane. Electrophoresis was performed at constant voltage of 125 V for 60 min followed by a constant voltage of 150 V for 30 min until the dye front reached the bottom of the gel. Pre-stained molecular weight markers included during electrophoresis (Bio-Rad Dual Color, cat # 161-0374, Hercules, CA) were used to verify electrotransfer of protein to the PVDF membrane and to estimate the molecular weight of the immunoreactive bands. Samples were electrotransferred to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) for 70 min at a constant current of 300 mA.

The membrane was then blocked by incubation in 5% (w/v) NFDM in 1× PBST for 30 minutes. The membrane was first probed with 25 mL of a 1:4000 dilution of goat anti-

CP4 EPSPS serum [lot 6844572, prepared using *E. coli*-produced CP4 EPSPS protein reference standard APS lot 20-100017 as the antigen] in 1% (w/v) NFDM in PBST for one hour. Excess serum was removed using three 5-min washes with PBST. The membrane was finally probed with HRP-conjugated rabbit anti-goat IgG (Sigma Chemical Company, St. Louis, MO) at a dilution of 1:10000 in 1% (w/v) NFDM in PBST for 45 min and again excess HRP-conjugate was removed using three 5-min washes with PBST. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences) and exposed (15, 20, 30 sec., and 1 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Image Analysis of Blot Films

Image analysis of immunoreactive bands on blot films was conducted using a Bio-Rad model GS-710 calibrated imaging densitometer (Hercules, CA) equipped with Quantity One software Version 4.3.0. The level of signal for the principal band corresponding to the CP4 EPSPS protein detected in each lane was measured as band contour quantity (avg. band OD × band area in mm^2). The percent difference between the plant- and *E. coli*-produced CP4 EPSPS proteins was calculated as shown below:

$$\left|\frac{(E.coli\text{ - CP4 EPSPS})-(\text{Plant - CP4 EPSPS})}{(E.coli\text{ - CP4 EPSPS})}\right|\times 100$$

The average overall percent difference was calculated and the immunoreactivities of the plant-produced and reference proteins were judged to be equivalent if the overall average percent difference was ≤ 20%.

Molecular Weight and Purity Estimation – SDS-PAGE

Aliquots of stock solutions of the CP4 EPSPS from MON 88913 and reference standard protein were diluted with 5× Laemmli buffer (Laemmli, 1970) to a final concentration of 0.2 µg/µL. Molecular weight markers (Bio-Rad broad-range, cat # 161-0317, Hercules, CA) used to estimate the molecular weight of the CP4 EPSPS from MON 88913 were diluted to a final concentration per protein band of 0.1 µg/µL. The plant-produced protein was analyzed in duplicate at 1, 2, and 3 µg total protein per lane. The *E. coli*-produced protein was analyzed at 1 µg as a reference standard. All samples were heated at ~104°C for 4 min and applied to a pre-cast Tris-Glycine 4→20% polyacrylamide gradient 12-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 125 V for 15 min followed by a constant voltage of 170 V for 65 min until the dye front approached the bottom of the gel. Proteins were fixed in the gel by gentle shaking in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained (2 h) with Brilliant Blue G-Colloidal stain (Sigma Chemical Co., St. Louis, MO), destained ~30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 2 h.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-710 densitometer with the supplied Quantity One software (version 4.3.0, Hercules, CA). Molecular weight values supplied by the manufacturer were used to estimate the molecular weight of each observed band. All visible bands within each lane were quantified. For the plant-produced CP4 EPSPS protein, purity was estimated as the percent optical density of the ~43 kDa band relative to all bands detected in the lane. Molecular weight and purity were reported as an average of all three loadings containing the plant-produced CP4 EPSPS protein.

B.3. Protein Levels

Materials

Tissue samples analyzed in this study were produced under field conditions in 2002 alongside the materials for molecular and protein characterization (Appendix C, Section C.3.) and were grown from seed lot GLP-0203-12170-S. An *E. coli*-produced CP4 EPSPS protein standard (Monsanto lot # 20-100015) was used as a reference for analysis of CP4 EPSPS protein levels.

Characterization of the Materials

The identities of the field-produced tissues and cottonseed were confirmed by verifying the chain-of-custody documentation and the tissues were assayed prior to use by PCR analysis to confirm the presence or absence of MON 88913, as appropriate.

Summary of Field Design and Tissue Collection

MON 88913 and MON 88913(-) were grown at four field locations in the U.S in 2002: Baldwin County, Alabama; Tulare County, California; Clarke County, Georgia; and Hockley County, Texas. These field sites provided a range of environmental and agronomic conditions representative of locations where MON 88913 is expected to be produced commercially. At each site, four replicated plots of MON 88913 and MON 88913(-) were planted using a randomized complete block field design. Young leaf, overseason leaf (OSL)1, OSL2, OSL3, root, seed, and pollen tissues were collected from each replicated plot at all field sites (Appendix C). Throughout the field production process, sample identity was maintained using unique sample identifiers and proper chain-of-custody documentation. Upon collection, all tissue samples were placed in uniquely labeled bags or containers. All tissue samples, with the exception of seed (which was stored and shipped at ambient temperature), were stored on dry ice and shipped frozen on dry ice to Monsanto, and stored at -80°C.

Young leaf samples were collected at the first true leaf growth stage from all field locations. The first fully expanded true leaves were nonsystematically collected from each MON 88913 and MON 88913(-) plot and all leaves from a given plot were pooled. Overseason leaf (OSL) samples were collected from the newest fully expanded leaf from each MON 88913 and MON 88913(-) plot from all field locations at the following time-points: OSL1 at approximately 4th node; OSL2 at approximately 50% white flower; and OSL3 at approximately cut-out. Root samples were collected from each MON 88913 and

MON 88913(-) plot at all field locations. The root was removed at the soil line and thoroughly washed with water to remove excess soil. The root samples were collected at approximately 50% white flower growth stage. Pollen samples were collected from each MON 88913 and MON 88913(-) plot at all field locations. Pollen was collected at approximately 50% white flower stage. Because of the limited quantity of cotton pollen, MON 88913 and MON 88913(-) pollen were collected and pooled across replicates at each site to generate sufficient quantities of samples. Seed samples were collected from each MON 88913 and MON 88913(-) plot at all field locations. The seed was harvested at crop maturity and all seed was ginned and delinted prior to sample processing.

Tissue Processing and Protein Extraction

During the processing step, dry ice was combined with the samples (except pollen) and vertical cutters or mixers were used to thoroughly grind and mix the tissues. Processed tissue samples were transferred into 15 ml tubes. All tissue samples were stored in a -80°C freezer prior to, and during the study. The CP4 EPSPS protein was extracted from cotton tissues following standard procedures. Extraction parameters for each tissue type and ELISA validations are described below. All tissues were extracted using a Harbil Mixer and insoluble material was removed from leaf, root, and pollen extracts by a Serum Filter System (Fisher Scientific, Pittsburgh, PA). Insoluble material was removed from seed extracts by centrifugation. The clarified extracts were divided and stored frozen in a -80°C freezer until ELISA analyses. During validation, extraction efficiency for each tissue type was determined by successive extraction of three replicates, where the last extraction employed a harsh buffer (e.g., 2X Laemmli buffer). To evaluate the analytical accuracy of the ELISA, extracts prepared from each tissue type of conventional cotton plants were spiked with known quantities of CP4 EPSPS protein at three concentrations spanning the range of the standard curve. The intra- and inter-assay precision were assessed by determining the coefficient of variation (CV) of the concentration of CP4 EPSPS protein measured for the positive control sample from 10 or more independent ELISAs using one-way analysis of variance (ANOVA). The limits of quantitation (LOQ) were calculated based on the lowest standard concentration. The ng/ml value was converted to µg/g fwt using the respective dilution factor and tissue-to-buffer ratio. The limits of detection (LOD) were calculated as the mean value using the data generated on conventional sample extracts for each tissue type plus three standard deviations. The LOD value in ng/ml was converted to µg/g fwt using the respective dilution factor and tissue-to-buffer ratio. The CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of CP4 EPSPS extraction buffer (TBA) and shaking in a Harbil mixer. The TBA buffer consisted of 100 mM Tris-base, 100 mM $Na_2B_4O_7 \cdot 10H_2O$, 10 mM $MgCl_2$, 0.05 % (v/v) Tween-20 at pH 7.8, and 0.2 % (w/v) L-ascorbic acid.

The positive quality control (QC) sample was prepared from cotton tissue that contained the CP4 EPSPS protein. The negative quality control sample was prepared from conventional cotton tissue that does not contain the *cp4 epsps* coding sequence and therefore does not produce the CP4 EPSPS protein. Extracts of the positive and negative QC samples were analyzed on every plate in triplicate wells. All positive QC samples fell within the range established during method validation and all negative QC samples

were less than the assay LOQ, as expected. Validation of the ELISA method establishes the specificity of the serum/antibody for the CP4 EPSPS protein.

ELISA Reagents

CP4 EPSPS protein standard for the antigen was produced by fermentation in *E. coli.* The protein was purified to greater than 90% purity by a combination of cell extraction, ammonium sulfate precipitation, hydrophobic and anion exchange chromatography. The purity-corrected total protein concentration of the purified standard was 3.7 mg/ml by amino acid composition analysis. The purity was 97% as determined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis and densitometric analysis. Mouse monoclonal antibody clone 39B6 (IgG2a isotype, kappa light chain; lot # 6199732) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography. The concentration of the purified IgG2a was determined to be 3.2 mg/ml by spectrophotometric methods. Production of the 39B6 monoclonal antibody was performed by TSD Bioservices, Inc. (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 0.02 M $Na_2HPO_4 \cdot 7H_2O$, 0.15 M NaCl, and 15 ppm ProClin 300 (Sigma Chemical Company, St. Louis, MO). The detection reagent was goat anti-CP4 EPSPS antibody (Sigma Chemical Company, St. Louis, MO) conjugated to HRP.

CP4 EPSPS ELISA Method

The CP4 EPSPS ELISA was performed using an automated robotic workstation (Tecan, Research Triangle Park, NC). Mouse anti-CP4 EPSPS antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) and immobilized onto 96-well microtiter plates at 1.0 µg/ml followed by incubation in a 4°C refrigerator for ≥ 8 h. Plates were washed in 1X PBS with 0.05% (v/v) Tween-20 (1X PBST) and blocked with the addition of 10% (w/v) non-fat dry milk in TBA. Plates were washed as before followed by the addition of 100 µl per well of CP4 EPSPS protein standard or sample extract and incubated at 37°C for 1 h. Plates were washed as before followed by the addition of 100 µl per well of goat anti-CP4 EPSPS peroxidase conjugate and incubated at 37°C for 1 h. Plates were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'- tetramethyl-benzidine (Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of CP4 EPSPS protein levels was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged in concentration from 0.456 - 14.6 ng/ml.

Moisture Analysis

Young leaf, overseason leaf, and root tissues were analyzed for moisture content using an IR 200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). Covance Laboratories Inc. (Madison, WI) analyzed seed tissue for moisture content. Because of limited sample quantity, moisture was not determined for pollen. A homogeneous TSSP was prepared by mixing approximately equal portions of the respective tissue type from each MON 88913 and MON 88913(-) plot within each field site. These pools were prepared for all tissues analyzed in this study (except pollen). The mean percent moisture for each TSSP was calculated from three analyses of a given pool and used to convert the

fwt protein levels at each site to dwt protein levels. A tissue-specific DWCF was calculated for each site as follows:

$$DWCF = 1 - [Mean\ Percent\ TSSP\ Moisture / 100]$$

The DWCF was only applied to samples with protein levels greater than the assay LOQ. All protein levels calculated on a fwt basis were converted into protein levels reported on a dwt basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ in\ Fresh\ Weight)}{(DWCF)}$$

Data Analyses

All ELISA plates were analyzed on a SPECTRAFluor Plus microplate reader (Tecan, Research Triangle Park, NC) using dual wavelengths. The CP4 EPSPS protein absorbance readings were determined at a wavelength of 450 nm with a simultaneous reference reading of 620 nm that was subtracted from the 450 nm reading. Data reduction analyses were performed using Molecular Devices SOFTmax PRO version 2.4.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fwt" basis. This conversion utilized the sample dilution factor and tissue-to-buffer ratio. The protein values in µg/g fwt were also converted to "µg/g dwt" by applying the DWCF. The arithmetic mean, SD, and range (fwt and dwt) were calculated for each tissue type across sites. Microsoft Excel 2000 (Version 9.0.4402 SR-1, Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein levels in MON 88913 tissues.

B.4. Phenotypic Evaluation

B.4.1. Seed Dormancy

Materials

Seed materials were produced in 2002 at three locations, Baldwin County, Alabama; Tulare County, California; and Clarke County, Georgia (Appendix C, Section C.3.). Seed materials included MON 88913, MON 88913(-), and two reference conventional cotton varieties from each production location. The cottonseed derived from MON 88913(-) provided values for dormancy and germination characteristics to which cottonseed from MON 88913 was compared. The reference cottonseed were commercially available conventional cotton varieties DP 90, DP 5690, Stoneville 474, Phytogen 72, Fibermax 989, and PSC 355. The reference materials provided a range of background values for dormancy and germination characteristics common to commercial cotton.

Characterization of the Materials

The identities of the field-produced cottonseed were confirmed by verifying the chain-of-custody documentation. The cottonseed were assayed prior to use by PCR analysis to confirm the presence or absence of MON 88913, as appropriate.

Performing Facility and Experimental Methods

Personnel at a certified seed-testing laboratory conducted the seed germination analysis. The facility was qualified to conduct seed germination tests consistent with the standards established by the AOSA, (AOSA, 1983; AOSA, 1998; AOSA, 1999). These testing guidelines provide an appropriate method to determine seed dormancy and germination characteristics. Seed was shipped to the performing laboratory and stored under ambient conditions until used. The experiment was conducted in temperature-controlled growth chambers using rolled towel tests to measure dormancy and germination characteristics. Four replicates of MON 88913, MON 88913(-), and two commercial reference varieties, each grown at three production locations, were tested in seven temperature regimes ranging from 10 to 40ºC. Prior to initiation and after completion of the experimental phase, seven temperature recorders were compared to a National Institute of Standards and Technology (NIST) certified glass thermometer at 10 and 20°C. The principal investigator at the performing laboratory confirmed that the thermometer and all temperature recorders displayed the same temperature reading.

Each of the seven growth chambers were maintained dark under one of the following temperature regimes:

- Constant target temperature of approximately 10, 20, 30, or 40°C
- Alternating target temperatures of approximately 10/20, 10/30, or 20/30°C

In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours. The temperature inside each growth chamber was monitored and recorded every 15 minutes using Watchdog™ 110 Data Loggers (with an accuracy of +/- 0.7°C). Temperature variation of +/- 3°C for less than one hour was considered acceptable. Each temperature regime was considered an experimental block. Rolled germination towels containing exactly 100 seed of each sample were prepared according to standards established by AOSA (1998).

To ensure material isolation during the preparation process, the germination towels for a given seed material (MON88913, MON 88913(-), or reference) were assembled (towel, seed, and water) for all temperature regimes and replications at a single workstation. After all germination towels were assembled for a given seed material, the workstation was thoroughly cleaned prior to assembling the germination towels for the next seed material.

Four replications of these towels were placed into each of seven growth chambers. Each towel was uniquely identified with its sample identification number, temperature regime, and replication number. For rolled towel germination testing, AOSA (1998) recommends

cotton be tested under the optimal temperature regime of 20/30°C. Therefore, each rolled germination towel in the 20/30°C temperature regime was checked for normal germinated, abnormal germinated, dead, firm swollen (viable and nonviable), and hard (viable and nonviable) seed were evaluated using AOSA definitions:

- Normal germinated seedlings exhibit normal developmental characteristics including a shoot in proportion to the roots, the presence of primary and secondary roots, and intact cotyledons with little visible damage.

- Abnormal germinated seedlings lack a shoot or root or appeared diseased with lesions on the cotyledons or shoot.

- Dead seed are visibly deteriorated and have become soft to the touch. Firm swollen seed have imbibed water and are easily cut by hand with a razor.

- Hard seed have not imbibed water and are hard to cut by hand with a razor.

In the non-optimal temperature regimes (10, 20, 30, 40, 10/20, and 10/30°C), cotton germinates at different rates and cannot be evaluated according to the same definitions. In particular, it is difficult to distinguish abnormal germinated seedlings from normal germinated seedlings that are developing at a slower rate. Therefore, these two categories were combined and referred to as germinated seedlings. Using AOSA requirements as a guide, each rolled towel in the non-AOSA temperature regimes were checked for germinated, dead, firm swollen (viable and nonviable), and hard seed (viable and nonviable), and were evaluated according to the following definitions:

- Germinated seedlings possess a radicle that extends at least 2 mm beyond the seed coat.
- Dead seed are visibly deteriorated and have become soft to the touch.
- Firm swollen seed had imbibed water and are easily cut by hand with a razor.
- Hard seed did not imbibe water and are hard to cut by hand with a razor.

Observations were made on the 4th and 12th days after experimental phase initiation. On the 4th day, normal germinated (20/30°C), abnormal germinated (20/30°C), or germinated seed (all other temperatures) and dead seed were counted and removed. Firm swollen and hard seed were counted but left on the towel. On the 12th day, normal germinated (20/30°C), abnormal germinated (20/30°C), or germinated seed (all other temperatures) and dead seed were counted and removed. Remaining firm swollen and hard seed were distinguished by cutting with a razor and subjected to a tetrazolium test for evaluation of viability following AOSA guidelines (AOSA, 1999). Numbers of viable firm swollen, nonviable firm swollen, viable hard, and nonviable hard seed were recorded upon completion of the tetrazolium test. Any nonviable firm swollen or hard seed were added to the dead category prior to statistical analysis. Specimens counted and removed from each towel were placed in a designated container for proper devitalization. All waste plant materials produced were devitalized by freezing at –80ºC for at least 48 hours.

Statistical Analysis
Two analysis of variance computations were performed, one for the AOSA-recommended temperature regime and one for all other temperature regimes, according to a randomized complete block design using Statistical Analysis Software (SAS© Version 8.2, SAS Institute, Inc., Cary, NC). Nonviable hard and firm swollen seed were added to the dead seed prior to calculating percent dead seed. For the AOSA-recommended temperature regime, evaluation characteristics analyzed were percent normal germinated seed, percent abnormal germinated seed, percent dead seed, percent viable firm swollen seed, and percent viable hard seed. For all other temperature regimes, evaluation characteristics analyzed were percent germinated seed, percent dead seed, percent viable firm swollen seed, and percent viable hard seed. Cottonseed from MON 88913 and MON 88913(-) were compared independently for each combination of production location, temperature regime, and germination characteristic within each analysis. The minimum and maximum values of the references were identified to establish the range of reference values. No comparisons were made between temperature regimes or between production locations. Differences detected were statistically significant at $p \leq 0.05$. The model was fit to the data using the mixed model procedure (PROC MIXED) of SAS.

B.4.2. Materials and Methods: Field Phenotypic Analysis

Field trials were established at 14 locations (two letter site code in parenthesis): Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC). These fourteen locations provided a range of environmental and agronomic conditions representative of major U.S. cotton-growing regions where the majority of commercial production of MON 88913 is expected to occur. The field cooperators at each site were familiar with the growth, production and evaluation of the cotton characteristics.

Materials
The test plants were MON 88913. The control plants were MON 88913(-).

Characterization of the Materials
The identities of the field planting seed were confirmed prior to use by PCR analysis to confirm the presence or absence of MON 88913, as appropriate.

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USDA-APHIS Compliance

Field trials were conducted in accordance with regulations of USDA-APHIS. Movement and release of regulated seed materials were conducted under the following USDA-APHIS notification numbers (locations in parentheses):

#02-016-27n (AL)
#02-025-01n (FL and RL)
#02-025-08n (LB and NV)
#02-018-16n (MR)
#02-022-55n (PV)
#02-023-15n (UC)
#02-022-54n (BM)
#02-028-28n (KS)
#02-025-02n (LL and SV)
#02-004-11n (PL)
#02-025-07n (TF)

Field Plot Design

A paired split-plot design with four replications was used to establish the field experiments except at KS, NV, and PL, where a randomized complete block design was used. For the paired split-plot design, subplots were MON 88913 and MON 88913(-). Each subplot consisted of two or four cotton rows approximately 30 ft in length. Other biotech cotton materials, not part of the scope of this petition, were planted in separate plots within the larger field sites. After excluding the other cotton materials from the statistical analysis, the design effectively became a randomized complete block.

Planting and Field Operations

Agronomic practices used to prepare field sites were typical for each respective region. A description of the field plots is presented below and includes information on soil type, plot size, and planting details.

Field and planting information

Location	Soil Type	Row Spacing (in)	Seed Depth (in)	Seeding Rate (seed/ft)	Plot Size
AL	Silt loam	38	1.0	3.25	4 rows x 30 ft
BM	Silt loam	40	0.75	3.9	4 rows x 30 ft
FL	Loamy sand	38	0.75	4	4 rows x 30 ft
KS	Clay loam	38	1.2	4.3	4 rows x 30 ft
LB	Clay loam	40	1.5	5	4 rows x 30 ft
LL	Sandy loam	38	0.75	4	2 rows x 30 ft
MR	Sandy loam	48	0.5	5	4 rows x 31.3 ft
NV	Clay	40	1.5	4.3	4 rows x 30 ft
PL	Sandy clay loam	30	0.75	4.3	4 rows x 30 ft
PV	Silt loam	38	1.0	4	4 rows x 30 ft
RL	Sandy loam	36	0.75	3.4	4 rows x 30 ft
SV	Sandy clay loam	38	1.0	4.3	4 rows x 30 ft
TF	Loamy sand	36	0.75	5	2 rows x 30 ft
UC	Undetermined	38	0.75	4.3	4 rows x 30 ft

Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC)

Data Collection and Documentation

The cooperator at each site was provided a notebook to record personnel, experiment identification, field site, planting, and phenotypic and environmental data. The raw data, transcribed data and supporting documentation are retained in the Monsanto Regulatory Archive.

Phenotypic Observations

The description of the characteristics measured and the dates of important experimental events are listed in Section VII., Table VII-2.

Ecological Observations

Differential response to observed insect, disease and abiotic stressors (e.g., heat, drought and excess water) were evaluated at each site. The overall plot area was examined and any visually observable differences between MON 88913 and MON 88913(-) plots were recorded. Every insect, disease or abiotic stressor was not evaluated at each field location because of a lack of occurrence at some locations.

Statistical Analysis

Monsanto's Statistics Technology Center performed all statistical analyses using SAS (SAS Version 8.2, SAS Institute, Inc., Cary, NC). Statistical differences were assessed at the 5% level ($p \leq 0.05$). Analysis of variance tests for each field location were conducted according to a randomized complete block design with four replications. The first statistical analysis compared MON 88913 to MON 88913(-) within each location. The second statistical analysis compared MON 88913 to MON 88913(-) across locations. Linear contrast was used to compare each characteristic within locations and across locations.

B.4.3. Materials and Methods: Composition of Cottonseed

Materials

MON 88913, MON 88913(-) and conventional reference cottonseed were grown at four U.S. locations in 2002. The field-produced cottonseed were produced in 2002 alongside the materials for molecular and protein characterization, and protein level determinations (Appendix C). All cottonseed samples were ginned at the production locations and acid-delinted at the Food and Protein Research and Development Center at Texas A&M University prior to compositional analyses.

MON 88913(-), has background genetics representative of MON 88913 but does not contain the *cp4 epsps* coding sequence or produce the CP4 EPSPS protein. Cottonseed of sixteen conventional, commercial cotton varieties produced along side of MON 88913 and were used as references. The varieties and seed lot numbers were as follows:

Reference Variety	I. D. Code	Location	Seed Lot Number
Stoneville 474	12254	California	REF-0203-12254-S
Stoneville 580	12255	California	REF-0203-12255-S
DP 90	12256	Alabama	REF-0203-12256-S
DP 51	12257	Alabama	REF-0203-12257-S
DP 5690	12258	Alabama	REF-0203-12258-S
DP 5415	12259	Alabama	REF-0203-12259-S
GTO-Maxx A	12260	California	REF-0203-12260-S
Phytogen 72	12261	California	REF-0203-12261-S
Fibermax 989	12264	Georgia	REF-0203-12264-S
PSC 355	12265	Georgia	REF-0203-12265-S
GA 161	12266	Georgia	REF-0203-12266-S
HS 12	12267	Georgia	REF-0203-12267-S
Paymaster 330	12268	Texas	REF-0203-12268-S
Paymaster 2379	12269	Texas	REF-0203-12269-S
AFD Rocket	12270	Texas	REF-0203-12270-S
All-Tex Atlas	12271	Texas	REF-0203-12271-S

Analytical reference standards were used as appropriate for each analytical procedure.

Characterization of the Materials

The identities of the MON 88913, MON 88913(-), and reference cottonseed were verified prior to use by confirming the chain-of-custody documentation supplied with the samples collected from the field. Additionally, the identities of the field-produced cottonseed were confirmed by PCR analysis to confirm the presence or absence of MON 88913, as appropriate.

Field Trials

The analyzed cottonseed were produced in U.S. field trials in 2002 at four replicated sites. The randomized block trials were conducted in California, Georgia, Alabama, and Texas. These sites provided a variety of environmental conditions representative of regions where MON 88913 is expected to be grown commercially. At each site, MON 88913, MON 88913(-) and conventional reference cottonseed were planted in approximately 200 ft^2 plots in each of four replicated blocks. Sixteen different commercial cotton varieties were planted, four per site. Each plot was clearly marked with a unique lot number and plot number for identification. In accordance with commercial practice, all plants were allowed to pollinate openly within a plot. Cottonseed samples were collected from all plots at seed maturity. The seed cotton was ginned and acid-delinted. Plots were harvested and seeds were ginned and delinted in the following order: MON 88913(-), conventional reference varieties, and MON 88913. The seed was stored at ambient temperatures until it was homogenized with dry ice. After homogenization, the cottonseed was stored in a –20°C freezer until shipment to the analytical laboratory facility on dry ice. At the analytical facility, the samples were stored in a –20°C freezer until analysis.

Summary of Analytical Methods

Cottonseed samples from MON 88913, MON 88913(-), and commercial reference materials were shipped overnight on dry ice to Covance Laboratories Inc., Madison, Wisconsin, for compositional analyses. Analyses were performed using methods that are currently used to evaluate the nutritional quality of food and feed. Samples were analyzed for proximates (protein, fat, ash, and moisture), ADF, NDF, crude fiber, TDF, amino acids, fatty acids, cyclopropenoid fatty acids, vitamin E, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), gossypol (free and total), and aflatoxins. Carbohydrate and caloric levels were determined by calculation.

Control of Bias

The cottonseed was subjected to identical conditions at the field sites with respect to environmental conditions, harvesting, storage, and shipment. Cottonseed was ground thoroughly before use to minimize tissue bias. The order of compositional analyses of the samples was randomized to minimize assay bias.

Data Analysis

Composition data from Covance Laboratories Inc., containing individual values for each analysis, were reviewed at Monsanto Company. They then were transferred to Certus International where they were converted into the appropriate units and statistically analyzed. The following sixteen analytes with >50% of observations below the LOQ of the assay were excluded from statistical analysis: aflatoxins B1, B2, G1, and G2, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid. For 18:3 gamma linoleic acid, 26 of the 95 of the observations were below the LOQ and were assigned a value equal to one half of the LOQ prior to conducting statistical analyses. The SAS GLM procedure was applied to all data (MON 88913, MON 88913(-) and reference) to detect potential outliers in the dataset by screening studentized PRESS residuals. Line, site and replication effects were included in the model. Studentized PRESS residuals identifed the iron result in replicate 4 of MON 88913(-) from California and the vitamin E result in replicate 3 of reference variety HS12 from Georgia as outliers. However, as the identified observations were not the extreme values for these analytes, they were not excluded from the statistical analyses.

All component values, except moisture, were converted from a fresh weight basis into their respective units. Statistical analyses were conducted on the converted values for each component in the cottonseed using a mixed model analysis of variance for the five sets of comparisons: analysis for each of the four replicated trial sites (AL, CA, GA, and TX), and one for the combination of all four sites. A total of 53 components statistically were evaluated (the initial 69 analytes minus the 16 for which >50% of the observations were below the LOQ). A total of 265 comparisons were made, and there were 53 components with five statistical analyses each.

Individual replicated site analyses used the model:

$$Y_{ij} = U + T_i + B_j + e_{ij} ,$$

where Y_{ij} = unique individual observation, U = overall mean, T_i = line effect, B_j = random block effect, and e_{ij} = residual error.

Combined site analyses used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk} ,$$

where Y_{ijk} = unique individual observation, U = overall mean, T_i = line effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by line interaction effect, and e_{ijk} = residual error. MON 88913 was compared to MON 88913(-) to determine statistically significant differences at $p \leq 0.05$.

Compositional analysis data from the conventional commercial reference varieties were used to determine a range of the reference values for each compositional analysis

component. Additionally, the commercial reference variety data were used to develop population tolerance intervals. A tolerance interval is an interval with a specified degree of confidence that contains at least a specified proportion, p, of an entire sampled population for the parameter measured. For each component, tolerance intervals were calculated that were expected to contain, with 95% confidence, 99% of the values expressed in the population of commercial varieties. Because negative quantities are not possible, calculated lower tolerance bounds that were negative were set to zero. SAS (SAS Institute Inc., Cary, NC, USA) was used to generate all summary statistics and perform all analyses.

B.4.4. Materials and Methods: Reproductive Tolerance and Floral Phenotypic Characteristics

Plant culture
MON 88913, MON 88913(-), and Roundup Ready cotton were planted in five-gallon pots at the Texas A&M University greenhouse facilities in College Station, TX. Pots were thinned two weeks after planting to one plant per pot. Plants were maintained under controlled environment conditions, and watered and fertilized as needed. The genetic background of these plants was from the cotton variety Coker 312.

Treatments
Treatments consisted of glyphosate sequentially applied over the top of MON 88913 and Roundup Ready cotton at three different stages of growth. Glyphosate was applied using Roundup WeatherMAX herbicide. The rate of glyphosate used at each application was 1.5 lb ae/A. Plants were sprayed initially with glyphosate at the approximately four-leaf (node) stage, and the second and third glyphosate applications were made when plants averaged 8 and 12 leaves (nodes), respectively. Thus, the plants received a total of approximately six times the recommended over-the-top single application rate of 0.76 lb ae/A.

Parameters
Pollen availability was determined 12 hours after pollination by visually observing first position flowers under a stereomicroscope. Two evaluations were used for this assessment. In the first method, the degree of anther opening (anther dehiscence) was evaluated on a scale of 0 to 4 where 0 = 0% dehisced, 1 = 25% dehisced, 2= 50% dehisced, 3= 75% dehisced and 4 = 100% dehisced. In the second method, pollen availability was determined by counting the number of pollen grains attached to the stigmatic lobe with the highest apparent number of pollen grains.

Pollen viability was assessed for first position flowers using Alexander's stain and Brewbaker and Kwack's (B&K) procedures (Alexander, 1969; Alexander, 1980; Brewbaker and Kwack, 1963). Alexander's dye reacts with pollen protoplasm to produce a purple color, and with the cellulose in pollen cell walls to produce a green color. Aborted pollen stains green because it does not have protoplasm, while nonaborted pollen stains a deep purple (Alexander, 1980). The B&K procedure uses a nutrient-rich

artificial media for pollen germination, and classifies pollen as either germinated and nongerminated.

Stamen length (anther + filament) and staminal column height (including ovary) were measured. Uppermost anther height as a percent of the pistil length was calculated as follows: anther height = 100-[(pistil length – uppermost anther height)/pistil length]. Pollen deposition was also evaluated on a scale of 0 to 3, where 0 = no pollen attached, 1 = distribution over lower 2/3 of stigma, 2 = distribution over upper 2/3 of stigma and 3 = even distribution over entire stigma.

Experimental Design and Statistical Analysis

The experiment was established as a randomized complete block design with five replications. Each replication consisted of a single pot containing one plant. Characteristics were measured on six to nine flowers per plant per replication (*n* ranged from 30 to 42). Results were analyzed statistically using the GLM procedure of SAS at a significance level of $\alpha=0.05$.

Appendix C: Phenotypic Evaluation Results and Individual Field Tables

C.1. Individual Field Site Data for Seed Dormancy and Germination

C.2. Individual Field Site Data Supporting Section VII. Phenotypic Evaluations

C.3. Tables from MON 88913 Field Samples Production in 2002

C.4. Reproductive Tolerance and Floral Phenotypic Characteristics

Appendix C: Phenotypic Evaluation Results and Individual Field Tables

C.1. Individual Field Site Data for Seed Dormancy and Germination

Individual Site Seed Dormancy and Germination Results and Discussion
The cottonseed evaluated were produced during 2002 in: Baldwin County, AL; Tulare County, CA; Clarke County, GA (Table C-1 and Section C.3.). Cottonseed of MON 88913, MON 88913(-) and six conventional cotton varieties were compared.

Table C-1. Starting Seed Materials for the Dormancy and Germination Evaluation.

Substance Type	Monsanto ID/Variety	Production Location(s)
Test	MON 88913	AL, CA, GA
Control	MON 88913(-)	AL, CA, GA
Reference	DP 90	AL
Reference	DP 5690	AL
Reference	Stoneville 474	CA
Reference	Phytogen 72	CA
Reference	Fibermax 989	GA
Reference	PSC 355	GA

Percent Germinated Seed
Percent germinated seed was evaluated in the 10, 20, 30, 40 10/20, and 10/30°C temperature regimes. For seed produced at CA, no differences in percent germinated seed were detected in any temperature regime between MON 88913 and MON 88913(-). Combined site data tables are presented in the main body of the text, Section VII; individual site data tables presented below, (Tables C-2 though C-4). For seed from AL, there were no differences between MON 88913 and MON 88913(-) except in the 40°C temperature regime; MON 88913 had a lower percentage of germinated seed than MON 88913(-) (49.0 vs. 56.3% and was just outside the reference range 50-61%). MON 88913 from the GA location had a lower percentage of germinated seed than MON 88913(-) in the 20°C (40.8 vs. 49.3%), 40°C (39.3 vs. 51.0%), and 10/30°C (36.5 vs. 49.3%) temperature regimes but performed within the range of the references in all cases. Across most temperature regimes, percent germination values for seed materials from the AL and GA locations were approximately half those of seed materials from the CA location. MON 88913, MON 88913(-) and reference seed were similarly affected. These results were not unexpected because cotton is commonly grown for lint, not seed, in the southeastern U.S. Humid conditions, typical of AL and GA, can degrade seed quality. Although seed quality at AL and GA was poor by seed production standards, it is representative of areas where MON 88913 will be grown for lint. Each of the germination differences occurred with seed from locations with reduced seed quality,

while no differences were detected in the high quality seed from the CA site. These germinated seed values were accompanied by corresponding increases in percent dead seed. Decreased germination accompanied by more dead seed, with no changes in hard or viable firm swollen seed, would not indicate an increased weed potential for MON 88913.

Percent Normal/Abnormal Germinated Seed

The percent normal germinated seed category was evaluated in the AOSA-recommended 20/30°C temperature regime. For the seed produced at the CA and GA locations, there were no differences detected between MON 88913 and MON 88913(-) for this characteristic. For the seed produced at AL, MON 88913 had a lower percentage of normal germinated seed than MON 88913(-) (46.3 vs. 55.5%); however, MON 88913 was within the range of the references. A single difference with no concurrent trend across production locations is most likely due to random experimental error and not as a result of altered germination characteristics of the seed. Percent abnormal germinated seed was also only evaluated in the AOSA-recommended 20/30°C temperature regime. No differences were detected between MON 88913 and MON 88913(-) for percent abnormal germinated seed. The lack of differences between MON 88913 and the MON 88913(-) for this characteristic supports a conclusion of no increased weed potential for MON 88913.

Percent Viable Hard Seed

No viable hard seed were observed in any seed materials (MON 88913, MON 88913(-), or reference) and no differences were detected among seed from any production location or temperature regime. The lack of differences in hard seed, a mechanism of seed dormancy, between MON 88913 and MON 88913(-) indicates that dormancy mechanisms in the seed have not been altered.

Percent Viable Firm Swollen Seed

No differences in percent viable firm swollen seed were detected between MON 88913 and MON 88913(-) for seed from AL and CA. In seed from the GA location, there was one difference detected in percent viable firm swollen seed in the 10/20°C temperature regime: MON 88913 had fewer viable firm swollen seed than MON 88913(-) (0.0 vs. 2.9%). This difference was small and unlikely to be biologically meaningful.

Percent Dead Seed

No differences in percent dead seed were detected between MON 88913 and MON 88913(-) in six temperature regimes for seed from the CA location, five temperature regimes for seed from the AL location, or four temperature regimes for seed from the GA location. For seed produced at the CA location, a single difference was detected in the 10/20°C temperature regime between MON 88913 and MON 88913(-) (3.3 vs. 7.7%); however, the response of MON 88913 was within the range of the responses observed from the reference materials that are representative of conventional cotton. Two differences were recorded between MON 88913 and MON 88913(-) in percent dead seed from AL at 40°C (51.0 vs. 43.8%) and 20/30°C (51.0 vs. 39.0%). For both of these differences, MON 88913 performed outside the range of the references (36-47% at

20/30°C and 39-50% at 40°C). Three differences were detected in percent dead seed for seed from GA at 20°C (59.3 vs. 50.8%), 40°C (60.8 vs. 49.0%), and 10/30°C (63.5 vs. 50.8%). For these three differences, the response of MON 88913 was within the range of responses for the references. All but one of these differences described above occurred in the locations with reduced seed quality. These differences, accompanied by corresponding decreases in germination, were likely a seed quality issue and do not indicate a change in the germination characteristics of the MON 88913.

Conclusions for Seed Dormancy

Out of 87 comparisons between MON 88913 and MON 88913(-), 75 were not statistically significant at $p \leq 0.05$. No differences were detected in percent viable hard seed or in percent abnormal germinated seed. Of the 12 significant differences, ten occurred in seed from production locations with reduced seed quality as determined by the low percentage of germinated seed of MON 88913(-) and the references, even at the optimal germination temperature. Cottonseed of MON 88913 from these locations showed reduced germination (five differences) when compared to MON 88913(-). In all cases, there was an accompanying rise in the number of dead seed (five differences). Decreased germination accompanied by more dead seed with no changes in hard or viable firm swollen seed, would not indicate increased weed potential of MON 88913. The remaining two statistical differences were detected between MON 88913 and MON 88913(-) in the 10/20°C temperature regime for percent dead seed (CA) and percent viable firm swollen seed (GA). These differences were small and unlikely to be of biological significance. The lack of differences in hard seed between MON 88913 and MON 88913(-) indicated that dormancy mechanisms in the seed are unchanged. These data support a conclusion of no change in the weed potential as a result of increased dormancy between MON 88913 compared to MON 88913(-). Furthermore, the data also support the familiarity/equivalence of MON 88913 to MON 88913(-).

Table C-2. Germination of Cottonseed Produced at the CA Location.†

Temp. Regime (°C)	Seed Material	Mean[1a]				
		Normal Germinated (%)	Abnormal Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
20/30[3]	MON 88913	96.0	1.8	2.3	0.0	0.0
	MON 88913(-)	95.5	2.8	1.8	0.0	0.0
	Reference Range[2]	82-94	1-6	0-12	0-0	0-2

Temp. Regime (°C)	Seed Material	Mean[1b]			
		Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
10	MON 88913	0.0	6.5	0.0	93.5
	MON 88913(-)	0.0	5.8	0.0	94.3
	Reference Range[2]	0-0	7-54	0-0	46-93
10/20[3]	MON 88913	95.4	3.3*	0.0	1.3
	MON 88913(-)	91.5	7.7	0.0	0.8
	Reference Range	81-95	3-13	0-0	2-7
20	MON 88913	97.3	2.8	0.0	0.0
	MON 88913(-)	96.3	3.5	0.0	0.3
	Reference Range	91-96	0-9	0-0	0-4
30	MON 88913	98.5	1.5	0.0	0.0
	MON 88913(-)	96.3	3.8	0.0	0.0
	Reference Range	88-95	5-12	0-0	0-0
40	MON 88913	91.3	8.8	0.0	0.0
	MON 88913(-)	88.8	11.3	0.0	0.0
	Reference Range	70-89	11-30	0-0	0-1
10/30[3]	MON 88913	98.0	2.0	0.0	0.0
	MON 88913(-)	95.8	4.3	0.0	0.0
	Reference Range	89-98	2-11	0-0	0-2

* Indicates a significant difference between MON 88913 and MON 88913(-) at $p \leq 0.05$.

† Seed used in these tests were produced during 2002 at Tulare County, CA.

[1a] Mean percent normal germinated, abnormal germinated, dead, viable firm swollen, or viable hard seed.

[1b] Mean percent germinated, dead, viable firm swollen, or viable hard seed.

[2] Minimum and maximum values of combined data for all reference varieties.

[3] In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours.

Table C-3. Germination of Cottonseed Produced at the GA Location.†

Temp. Regime (°C)	Seed Material	Mean[1a]				
		Normal Germinated (%)	Abnormal Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
20/30[3]	MON 88913	30.0	8.3	61.7	0.0	0.0
	MON 88913(-)	37.0	8.5	54.5	0.0	0.0
	Reference Range[2]	15-29	3-18	60-74	0-0	0-0

Temp. Regime (°C)	Seed Material	Mean[1b]			
		Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
10	MON 88913	0.0	67.3	0.0	32.7
	MON 88913(-)	0.0	69.3	0.0	30.8
	Reference Range[2]	0-0	68-97	0-0	3-32
10/20[3]	MON 88913	38.3	61.7	0.0	0.0*
	MON 88913(-)	41.7	55.4	0.0	2.9
	Reference Range	24-37	62-74	0-0	0-2
20	MON 88913	40.8*	59.3*	0.0	0.0
	MON 88913(-)	49.3	50.8	0.0	0.0
	Reference Range	24-50	50-76	0-0	0-0
30	MON 88913	40.3	59.8	0.0	0.0
	MON 88913(-)	46.3	53.8	0.0	0.0
	Reference Range	18-47	53-82	0-0	0-0
40	MON 88913	39.3*	60.8*	0.0	0.0
	MON 88913(-)	51.0	49.0	0.0	0.0
	Reference Range	22-44	56-78	0-0	0-0
10/30[3]	MON 88913	36.5*	63.5*	0.0	0.0
	MON 88913(-)	49.3	50.8	0.0	0.0
	Reference Range	18-37	63-82	0-0	0-0

* Indicates a significant difference between MON 88913 and MON 88913(-) at $p \leq 0.05$.

† Seed used in these tests were produced during 2002 at Clarke County, GA.

[1a] Mean percent normal germinated, abnormal germinated, dead, viable firm swollen, or viable hard seed.

[1b] Mean percent germinated, dead, viable firm swollen, or viable hard seed.

[2] Minimum and maximum values of combined data for all reference varieties.

[3] In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours.

Table C-4. Germination of Cottonseed Produced at the AL Location.†

Temp. Regime (°C)	Seed Material	Mean[1a]				
		Normal Germinated (%)	Abnormal Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
20/30[3]	MON 88913	46.3*	2.7	51.0*	0.0	0.0
	MON 88913(-)	55.5	5.5	39.0	0.0	0.0
	Reference Range[2]	45-57	1-11	36-47	0-0	0-0

Temp. Regime (°C)	Seed Material	Mean[1b]			
		Germinated (%)	Dead (%)	Viable Hard (%)	Viable Firm Swollen (%)
10	MON 88913	0.0	62.8	0.0	37.3
	MON 88913(-)	0.0	46.5	0.0	53.5
	Reference Range[2]	0-0	37-67	0-0	33-63
10/20[3]	MON 88913	53.7	45.5	0.0	0.0
	MON 88913(-)	56.9	42.8	0.0	0.3
	Reference Range	49-65	34-48	0-0	0-3
20	MON 88913	56.3	43.8	0.0	0.0
	MON 88913(-)	54.8	45.3	0.0	0.0
	Reference Range	54-65	35-46	0-0	0-0
30	MON 88913	56.3	43.8	0.0	0.0
	MON 88913(-)	55.4	44.6	0.0	0.0
	Reference Range	49-66	34-51	0-0	0-0
40	MON 88913	49.0*	51.0*	0.0	0.0
	MON 88913(-)	56.3	43.8	0.0	0.0
	Reference Range	50-61	39-50	0-0	0-0
10/30[3]	MON 88913	55.3	44.8	0.0	0.0
	MON 88913(-)	58.5	41.5	0.0	0.0
	Reference Range	54-78	22-46	0-0	0-0

* Indicates a significant difference between MON 88913 and MON 88913(-) at $p \leq 0.05$.

† Seed used in these tests were produced during 2002 at Baldwin County, AL.

[1a] Mean percent normal germinated, abnormal germinated, dead, viable firm swollen, or viable hard seed.

[1b] Mean percent germinated, dead, viable firm swollen, or viable hard seed.

[2] Minimum and maximum values of combined data for all reference varieties.

[3] In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours.

C.2. Individual Field Site Data Supporting Section VII. Phenotypic Evaluations

Individual Field Site Plant Growth and Development Results and Discussion
Comparisons of phenotypic parameters between MON 88913, MON 88913(-), and conventional cotton were conducted to establish the phenotypic and seed compositional equivalence of MON 88913. Combined site data and tables are presented in the main body of the text, Section VII; individual site data tables are presented below. In each of these assessments, MON 88913 was compared to MON 88913(-). Fourteen field locations were used for the assessments during 2002:

Location	Location Code
Rapides Co., Louisiana	AL
Limestone Co., Alabama	BM
Florence Co., South Carolina	FL
Mississippi Co., Arkansas	KS
Lubbock Co., Texas	LB
Washington Co., Mississippi	LL
Pinal Co., Arizona	MR
Fort Bend Co., Texas	NV
San Patricio Co., Texas	PL
Pemiscot Co., Missouri	PV
Edgecombe Co., North Carolina	RL
Oktibbeha Co. Mississippi	SV
Tift Co., Georgia	TF
Obion Co., Tennessee	UC

Growth and Development
There were no differences at any location between the test MON 88913 and MON 88913(-) for six of 11 plant growth and development characteristics measured. Single differences at single sites were detected between MON 88913 and MON 88913(-) for the five remaining plant growth and development characteristics (Tables C-5, C-6). One difference was detected at the LB location, where there was a difference between MON 88913 and MON 88913(-) in the number of emerged plants at the first emergence count. This difference was not detected at the other locations and was attributed to the time at which the data were recorded. The cooperator collected these data at seven days after planting (DAP) when relatively few plants had emerged. By the second emergence count, there was no detected difference in plant emergence between MON 88913 and MON 88913(-). One difference was detected at PL where MON 88913 had a greater number of emerged plants at the second count than MON 88913(-) (229 vs. 213 plants per 30 ft, respectively). At TF, MON 88913 was taller than MON 88913(-) at the third height measurement (114 vs. 102 cm, respectively). One difference in days until 50% flowering was detected at BM where MON 88913 developed more slowly than

MON 88913(-) (74 vs. 69 DAP, respectively). Yield of MON 88913 was greater than MON 88913(-) at UC (1856 vs. 1567 lbs/acre, respectively).

A consistent trend toward increased emergence or yield would be agronomically desirable, but could indicate increased weed potential if the trait were transferred to a wild relative. However, no consistent trends for changes in these specific characteristics were observed across locations (Table VII-3). Thus, they likely are due to random experimental effects and are unlikely to be biologically meaningful in terms of plant weed potential of the crop itself, or if the trait were transferred to a wild relative. In the pooled analysis across locations, there was one difference detected between MON 88913 and MON 88913(-) for one of the 11 characteristics (Table VII-3). The date until 50% flowering was later for MON 88913 than MON 88913(-) (64 vs. 63 DAP, respectively). A time-by-location interaction was detected for this characteristic (data not shown): at six locations the MON 88913 date until 50% flowering was later than MON 88913(-), while at three locations they were the same. Thus, MON 88913 on average flowered later than MON 88913(-). This difference was one day at most sites and therefore has no biological meaning with respect to plant pest potential.

Plant Map Data

Plant mapping is a technique used to identify boll position and other characteristics on a cotton plant. Plant map data were used to assess overall plant morphology and boll retention (Table C-7). There were no differences detected between MON 88913 and MON 88913(-) for 13 of 20 plant map characteristics. Single differences at single sites were detected between MON 88913 and MON 88913(-) for six plant map characteristics. One difference in plant height at mapping was detected at RL, where MON 88913 was shorter than MON 88913(-) (84 vs. 96 cm, respectively). At SV, height per node was lower in MON 88913 compared to MON 88913(-) (5.9 vs. 6.3 cm/node, respectively). The total bolls per 10 plants at SV, the number of position 2 bolls per 10 plants at KS and the percent position 2 bolls on nodes 4 to 9 at LB were lower in MON 88913 compared to MON 88913(-) (74 vs. 82 bolls; 9 vs. 14 total position 2 bolls/10 plants; 31 vs. 49% position 2 bolls on nodes 4 – 9, respectively). The percent first position bolls on nodes 10 – 14 at FL was greater on MON 88913 compared to MON 88913(-) (40 vs. 26%, respectively). Two differences were detected in number of nodes per plant at LB and PV. At LB, MON 88913 had more nodes than MON 88913(-) (17 vs. 15 nodes, respectively), while the opposite was observed at PV where MON 88913 had fewer nodes than MON 88913(-) (14 vs. 15 nodes, respectively).

Trends toward reduced plant height would not contribute to increased plant weed potential, while differences in boll retention may indicate an increase in weed potential of the crop itself or a receiving wild relative. However, no consistent trends for changes in these specific characteristics, or any of the other measured plant map characteristics, occurred when the data were pooled across locations (Table VII-4). Thus, the detected differences in the by-location analysis likely are due to random experimental effects and are unlikely to be biologically meaningful in terms of plant weed potential of the crop itself, or if the trait were transferred to a wild relative.

Boll/Seed Measurements

No differences were detected between MON 88913 and MON 88913(-) for two of four boll/seed measurements (Table C-8). Single differences at single sites were detected between MON 88913 and MON 88913(-) for two boll/seed measurements (Table C-8). Seed index of MON 88913 was lower than MON 88913(-) at UC (9.9 vs. 11.3 g per 100 seed, respectively). At PV, MON 88913 had more seed per boll than MON 88913(-) (29 vs. 26 seed per boll, respectively).

A consistent trend toward greater seed numbers may indicate an increase in weed potential if transferred to a wild relative. However, no consistent trends for changes in seed number occurred when the data were pooled across locations (Table VII-5). Thus, the difference in the by-location analysis for seed number likely was because of random experimental effects and is unlikely to be biologically meaningful in terms of plant weed potential of the crop itself, or if the trait were transferred to a wild relative. In the pooled analysis of the boll/seed measurements, there was one difference detected between MON 88913 and MON 88913(-) for seed index. The MON 88913 seed index was lower than the MON 88913(-) seed index (9.56 vs. 9.83 g per 100 seed, respectively). A test material by location interaction was also detected for this characteristic (data not shown): at seven locations the MON 88913 seed index was lower than MON 88913(-), at one location the MON 88913 seed index was not different from MON 88913(-), and at two locations the MON 88913 seed index was higher than MON 88913(-). On average, MON 88913 had a lower seed index than MON 88913(-). This difference was approximately 0.3 g per 100 seed, and likely has little biological meaning with respect to plant weed potential.

Boll and Fiber Quality Characteristics

There were no differences detected between MON 88913 and MON 88913(-) for three of six boll and fiber quality characteristics (Table C-9). Single differences at single sites were detected between MON 88913 and MON 88913(-) for two boll and fiber quality characteristics (Table C-9). At SV, MON 88913 had lighter bolls than MON 88913(-) (4.8 vs. 5.0 g per boll, respectively). Fiber micronaire for MON 88913 was lower than MON 88913(-) at the LB location (3.80 vs. 4.30 mike units, respectively). Differences in fiber elongation were detected at two locations. At BM, MON 88913 had lower percent elongation than MON 88913(-) (7.525 vs. 8.150%, respectively), while the opposite was observed at KS (8.438 vs. 7.688%, respectively). Changes in micronaire or percent elongation, while important for consideration of crop performance, have little impact on plant weed potential, and both micronaire values are agronomically equivalent, falling within the premium target range of 3.7 to 4.2.

When the boll and fiber quality data were pooled across locations, there were two differences detected between MON 88913 and MON 88913(-) for two of the six characteristics assessed (Table VII-6). Boll size of MON 88913 was smaller than MON 88913(-) (4.56 vs. 4.70 g per boll, respectively). In addition, MON 88913 micronaire was less than MON 88913(-) (3.758 vs. 3.881 mike units, respectively). Small changes in seed size and micronaire are unlikely to increase plant weed potential.

Insect, Disease and Abiotic Stressors

Each field site was rated four times during the season for specific insect pests, diseases and abiotic stressors, although not all sites were rated for each pest or stressor because of lack of occurrence (Table C-10). These qualitative data were not statistically analyzed. Fourteen insect categories (species or group), four disease categories and ten abiotic stressors were evaluated. Out of 106 insect observations, only one location reported a difference in susceptibility between MON 88913 and MON 88913(-) (Table C-10). Beet armyworm was a severe stressor at TF on the first observation date and the cooperator noted, that in one of the four replications, the MON 88913 plot had more damage than the MON 88913(-) plot (data not shown). This was not observed in the other three replications, at other observations times at this location, or at other locations. A differential response between MON 88913 and MON 88913(-) to slight Roundup herbicide drift was observed at UC. The cooperator observed herbicide damage in the MON 88913(-) plots. This was not unexpected because MON 88913(-) is not tolerant to Roundup agricultural herbicide and damage would occur if spray drift occurred. The plants recovered quickly, and no symptoms were observed at the second observation. Out of seven disease and 38 abiotic stressor observations, no differences were observed between MON 88913 and MON 88913(-). These results support the conclusion that the ecological interactions of cotton have not been altered in MON 88913.

Table C-5. Plant Emergence, Growth Rate and Vigor at 14 Locations in 2002.

Location[1]	1st Emergence Count (# per 30ft)		2nd Emergence Count (# per 30ft)		1st Height Measurement (centimeters)		1st Vigor[2] Rating		2nd Height Measurement (centimeters)		2nd Vigor[2] Rating		3rd Height Measurement (centimeters)		3rd Vigor[1] Rating	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
AL	104	104	100	100	23	23	7	8	83	84	7	8	116	114	7	7
BM	110	111	104	98	14	14	7	7	50	49	8	8	106	103	-	-
FL	32	46	96	96	5	5	8	8	41	39	8	8	68	74	9	9
KS	127	122	138	130	25	25	-	-	81	78	-	-	88	92	-	-
LB	2*	14	119	119	8	7	6	6	24	24	10	9	55	48	10	10
LL	111	108	124	121	10	10	10	10	58	60	10	10	116	116	10	10
MR	-	-	99	108	-	-	-	-	-	-	-	-	-	-	-	-
NV	110	110	109	111	53	47	9	9	103	102	9	9	-	-	-	-
PL	174	174	229*	213	26	24	7	7	71	68	9	9	94	89	10	10
PV	120	124	127	127	-	-	5	5	-	-	6	6	-	-	6	6
RL	-	-	-	-	9	11	8	9	31	35	10	10	77	81	7	7
SV	109	110	110	110	-	-	-	-	78	82	-	-	-	-	-	-
TF	103	117	252	256	24	26	10	10	77	79	9	9	114*	102	10	10
UC	114	111	108	107	-	-	-	-	-	-	-	-	72	70	10	10

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

[2]Rated on a scale of 1-10, where 1 = poor and 10 = very good vigor

Table C-6. Plant Development Characteristics at 14 Locations During 2002.

Location[1]	Days until 50% flowering DAP		Node Above Cracked Boll DAP		Seedcotton Yield lbs/acre	
	Test	Control	Test	Control	Test	Control
AL	58	57	-	-	711	872
BM	74*	69	-	-	2932	3068
FL	-	-	118	117	671	568
KS	66	65	125	125	2241	2201
LB	64	63	104	102	1987	1805
LL	63	63	125	127	3336	3097
MR	-	-	-	-	1751	1718
NV	69	68	-	-	3485	3158
PL	60	59	111	111	2744	2722
PV	-	-	-	-	2926	2989
RL	66	66	122	120	1041	968
SV	-	-	-	-	-	-
TF	61	61	126	126	3261	3025
UC	-	-	116	116	1856*	1567

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-7. End of Season Plant Map Data from 14 Locations During 2002.

Location[1]	Height at Plant Mapping (centimeters)		Nodes (number)		Height per Node (centimeters)		Total Bolls (# per 10 plants)		Total Position 1 Bolls (# per 10 plants)		Total Normal Position 1 Bolls (# per 10 plants)		Total Abnormal Position 1 Bolls (# per 10 plants)	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BM	99	101	17	17	6.0	5.9	71	79	48	52	48	52	0	0
FL	67	67	16	16	4.3	4.3	43	42	28	30	28	30	0	0
KS	96	96	19	19	5.1	5.0	51	54	43	41	38	37	5	4
LB	49	45	17*	15	3.0	3.1	42	36	29	23	28	22	1	1
LL	114	115	20	21	5.6	5.5	124	129	91	87	91	87	0	0
MR	56	53	37	34	1.5	1.5	79	82	53	59	49	55	4	4
NV	120	118	19	20	6.3	6.0	96	86	55	50	49	47	6	3
PL	103	99	21	21	4.9	4.8	64	68	46	43	43	42	2	2
PV	-	-	14*	15	-	-	84	99	57	63	57	63	0	0
RL	84*	96	20	22	4.1	4.3	49	52	41	41	35	35	6	6
SV	123	131	21	21	5.9*	6.3	74*	82	53	54	52	53	1	0
TF	107	106	18	18	5.9	6.1	89	82	61	58	58	55	3	3
UC	33	31	15	16	2.1	2.0	69	69	49	47	49	46	0	1

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-7 (Continued). End of Season Plant Map Data from 14 Locations During 2002.

Location[1]	Total Position 2 Bolls # per 10 plants		Total Normal Position 2 Bolls # per 10 plants		Total Abnormal Position 2 Bolls # per 10 plants		Vegetative Bolls # per plant		Abnormal Bolls %	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-	-	-
BM	23	27	23	27	0	0	2	2	0	0
FL	16	13	16	13	0	0	1	1	0	0
KS	9*	14	7	12	2	2	0	1	12.25	10.25
LB	13	13	12	13	1	0	1	0	5.5	4.5
LL	32	42	32	42	0	0	0	1	0	0
MR	26	23	23	21	3	2	1	1	9.5	7.25
NV	41	37	40	36	1	1	1	1	6.5	4.25
PL	18	25	16	23	2	2	-	-	7.5	5
PV	28	36	28	36	0	0	1	1	0	0
RL	8	11	7	8	1	3	0	0	14.5	17.75
SV	21	28	21	28	0	0	0	0	0.75	0.5
TF	28	24	27	23	1	1	1	1	5	5.25
UC	20	22	20	22	0	1	1	1	0	2

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-7 (Continued). End of Season Plant Map Data from 14 Locations During 2002.

Location[1]	Position 1 Bolls on Nodes 4 to 9		Position 1 Bolls on Nodes 10 to 14		Position 1 Bolls on Nodes 15 to 19		Position 1 Bolls on Nodes 20 to 26	
	% of nodes with bolls							
	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-
BM	52	37	54	56	41	48	-	10
FL	33	47	40*	26	6	8	-	-
KS	59	61	47	39	6	4	0	0
LB	48	43	28	20	5	1	0	0
LL	76	68	73	68	38	40	12	13
MR	55	61	35	40	44	41	31	32
NV	37	28	53	51	36	31	7	17
PL	36	39	35	40	22	13	8	3
PV	63	66	74	71	50	93	-	-
RL	64	74	28	14	2	0	0	0
SV	40	35	52	45	43	49	64	65
TF	63	56	49	54	59	58	83	-
UC	59	57	58	54	30	30	0	-

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-7 (Continued). End of Season Plant Map Data from 14 Locations During 2002.

Location[1]	Position 2 Bolls on Nodes 4 to 9		Position 2 Bolls on Nodes 10 to 14		Position 2 Bolls on Nodes 15 to 19		Position 2 Bolls on Nodes 20 to 26	
	% of nodes with bolls							
	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-
BM	56	40	39	39	46	22	-	-
FL	34	24	18	18	0	0	-	-
KS	18	37	20	19	25	0	-	0
LB	31*	49	48	46	75	-	-	-
LL	25	30	31	42	43	36	-	-
MR	36	28	27	20	10	18	13	4
NV	36	39	41	33	30	48	23	47
PL	29	30	30	25	25	24	7	8
PV	39	47	32	39	0	0	-	-
RL	19	26	9	0	0	0	0	0
SV	15	16	18	29	23	21	21	5
TF	21	20	35	32	24	16	0	-
UC	46	47	45	50	20	-	-	-

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-8. Boll/Seed Measurements from 14 Locations During 2002.

Location[1]	Seed Index g per 100 seed		Total Seed per boll # per boll		Mature Seed per boll # per boll		Immature Seed per boll # per boll	
	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-
BM	9.3	9.6	-	-	-	-	-	-
FL	8.0	8.0	-	-	-	-	-	-
KS	-	-	-	-	-	-	-	-
LB	9.5	9.8	-	-	-	-	-	-
LL	9.9	10.5	31	31	28	28	3	3
MR	10.8	10.9	27	25	26	24	1	1
NV	9.7	10.0	30	29	29	29	1	1
PL	10.3	10.0	33	33	30	29	3	4
PV	9.5	10.0	29*	26	26	24	3	2
RL	-	-	-	-	-	-	-	-
SV	-	-	-	-	-	-	-	-
TF	8.5	8.3	32	33	31	31	1	2
UC	9.9*	11.3	-	-	-	-	-	-

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

Table C-9. Boll and Fiber Quality Characteristics From 14 Locations During 2002.

Location[1]	Boll Size g/boll		Micronaire mike units[2]		Elongation %		Strength g/tex[2]		Span Length 2.5% inches		Span Length 50% inches	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
AL	-	-	-	-	-	-	-	-	-	-	-	-
BM	5.5	5.8	3.58	3.70	7.525*	8.150	19.225	19.950	1.180	1.185	0.603	0.588
FL	3.2	3.9	3.40	3.73	5.313	5.250	16.513	16.200	0.969	0.956	0.491	0.488
KS	4.2	4.5	3.60	3.60	8.438*	7.688	20.488	20.225	1.180	1.190	0.559	0.564
LB	4.5	4.5	3.80*	4.30	6.375	6.000	20.788	19.825	1.076	1.051	0.550	0.530
LL	4.9	5.2	3.83	3.75	7.250	7.125	20.175	21.525	1.206	1.205	0.591	0.610
MR	4.6	4.3	4.65	4.85	5.813	5.813	18.613	18.613	1.085	1.070	0.513	0.510
NV	4.7	4.8	4.20	4.28	5.563	5.625	20.288	20.225	1.135	1.136	0.585	0.594
PL	5.0	5.0	3.45	3.38	6.563	6.938	20.650	20.163	1.214	1.210	0.595	0.605
PV	4.4	4.2	3.43	3.60	6.900	7.175	19.125	20.175	1.165	1.165	0.543	0.545
RL	3.8	3.9	4.10	4.10	6.688	6.563	18.688	17.750	0.978	1.003	0.470	0.470
SV	4.8*	5.0	-	-	-	-	-	-	-	-	-	-
TF	4.5	4.8	3.58	3.70	8.563	8.313	20.625	20.413	1.165	1.175	0.553	0.558
UC	5.2	5.3	3.50	3.60	6.625	6.688	21.238	22.313	1.185	1.199	0.585	0.605

Test = MON 88913; Control = MON 88913(-).

*Indicates a difference was detected ($p \leq 0.05$) between MON 88913 and MON 88913(-) for a given characteristic within a location. Dashes indicate data not available.

[1]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

[2]Standard industry fiber quality characteristics. See details in Appendix A.

Table C-10. Insect, Disease and Abiotic Stressor Observations[1] During 2002.

			Level of Stressor			
Stressor	**Identity**	**Location[†]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**
Insect	Aphids	AL	none	none	none	none
		LL	none	none	none	none
		SV	none	-	-	-
	Beet armyworm	BM	-	-	slight	-
		FL	-	-	slight	mod
		TF	sev[2]	slight	mod	-
	Cabbage/Soybean Looper	PL	none	none	none	none
	Cotton bollworm	AL	none	slight	slight	none
		BM	-	-	slight	-
		LB	-	-	slight	-
		LL	none	mod	mod	mod
		PL	none	none	none	none
		RL	-	-	slight	slight
		TF	-	slight	mod	-
	Flea hoppers/Stink bugs	PL	none	none	none	none
		NV	-	mod	-	-
	Leaf perforator	PL	none	none	none	none
	Lepidoptera (unspecified)	NV	-	mod	-	-
	Lygus bugs	MR	none	none	-	slight
	Pink bollworm	MR	none	none	none	slight
	Stink bugs	LL	none	none	slight	mod
	Tarnished plant bugs	AL	none	slight	slight	none
		LL	none	mod	slight	slight
		SV	none	-	-	-
	Thrips	AL	none	none	none	none
		BM	slight	slight	-	-
		FL	mod	-	-	-
		LB	mod	-	-	-
		LL	slight	none	none	none
		MR	none	slight	slight	none
		RL	slight	-	-	-
		SV	none	-	-	-
		UC	slight	-	-	-
	Tobacco budworm	AL	none	slight	slight	none
		LL	none	mod	mod	mod
		PL	none	none	none	none
		RL	-	-	slight	slight
		TF	-	-	mod	-
	Whiteflies	AL	-	-	-	-
		MR	none	none	mod	sev
		TF	-	-	mod	-

Table C-10 (Continued). Insect, Disease and Abiotic Stressor Observations[1] During 2002.

			Level of Stressor			
Stressor	**Identity**	**Location[†]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**
Disease	Boll rot	RL	-	-	-	mod
	Pythium	RL	None	-	-	-
	Rhizoctonia	RL	None	-	-	-
	Verticillium	MR	None	none	none	none
Abiotic	Cavitation	UC	-	-	mod	
	Cold	BM	Mod	-	-	-
		RL	slight	-	-	-
	Crusting	TF	sev	-	-	-
	Drought	AL	slight	slight	slight	none
		BM	-	-	-	mod
		FL	sev	mod	sev	mod
		LB	-	slight	slight	-
		NV	sev	mod	-	-
		RL	none	mod	mod	none
		TF	slight	slight	none	-
		UC	-	sev	sev	-
	Flood	NV	-	-	-	mod
	Heat	FL	sev	sev	sev	sev
		TF	mod	sev	slight	-
	Herbicide	SV	-	-	mod	-
	Rapid growth	BM		slight	-	-
	Glyphosate drift[3]	UC	slight	-	-	-
	Weed competition[4]	TF	slight	-	-	-

[1]Each field site was rated approximately 4, 8, 12, and 16 weeks after planting for specific insect pests, diseases and abiotic stressors, although not all sites were rated for each pest or stressor because of lack of occurrence. Any observed visual differences between the response of MON 88913 and MON 88913(-) are footnoted. Mod = moderate, sev = severe (details of methods presented in Appendix A).

[2]In one of four replications the test material has more beet armyworm damage than the control. This was not observed in the other three replications.

[3]Slight glyphosate drift observed on some control plots; however, plants had grown out of symptoms by second observation.

[4]Slight weed competition observed across the entire plot area but controlled with subsequent herbicide applications.

[†]Field trials were established at 14 locations: Rapides Co., Louisiana (AL), Limestone Co., Alabama (BM), Florence Co., South Carolina (FL), Mississippi Co., Arkansas (KS), Lubbock Co., Texas (LB), Washington Co., Mississippi (LL), Pinal Co., Arizona (MR), Fort Bend Co., Texas (NV), San Patricio Co., Texas (PL), Pemiscot Co., Missouri (PV), Edgecombe Co., North Carolina (RL), Oktibbeha Co. Mississippi (SV), Tift Co., Georgia (TF), and Obion Co., Tennessee (UC).

C.3. Tables from MON 88913 Field Samples Production in 2002

In order to generate the materials for *in planta* CP4 EPSPS protein characterization and quantification, molecular characterization, cottonseed composition, and evaluation of cottonseed dormancy, replicated field production trials were conducted at four locations in the U.S. during 2002 (Table C-11). A description of the seed materials is presented in Tables C-12, C-13. Roundup UltraMAX herbicide treatments are presented in Table C-14. Tables C-15, C-16, and C-17 describe sample collections.

Table C-11. 2002 Field Sample Production Site Locations.

No.	Production Site	APHIS Notification
1	Baldwin County, Alabama	02-042-31n
2	Tulare County, California	02-042-31n
3	Clarke County, Georgia	02-042-31n
4	Hockley County, Texas	02-042-31n

Table C-12. MON 88913 and MON 88913(-) Planting Seed for 2002 Field Production.

Material Code	Seed Lot Number	Monsanto ID	Phenotype
12170	GLP-0203-12170-S	MON 88913	Glyphosate tolerant
12171	GLP-0203-12171-S	MON 88913(-)	Nongyphosate tolerant

MON 88913 and MON 88913(-) seed were planted at all production sites

Table C-13. Conventional (Reference) Planting Seed for 2002 Field Production.

Site Code[1]	Material Code	Seed Lot Number	Variety Name[2]
AL	12256	REF-0203-12256-S	Delta Pine - 90
AL	12257	REF-0203-12257-S	Delta Pine - 51
AL	12258	REF-0203-12258-S	Delta Pine - 5690
AL	12259	REF-0203-12259-S	Delta Pine - 5415
CA	12254	REF-0203-12254-S	Stoneville - 474
CA	12255	REF-0203-12255-S	Stoneville - 580
CA	12260	REF-0203-12260-S	GTO MAXXA
CA	12261	REF-0203-12261-S	Phytogen - 72
GA	12264	REF-0203-12264-S	FiberMax - 989
GA	12265	REF-0203-12265-S	Phytogen - PSC 355
GA	12266	REF-0203-12266-S	Phytogen - GA 161
GA	12267	REF-0203-12267-S	HS 12
TX	12268	REF-0203-12268-S	Paymaster 330
TX	12269	REF-0203-12269-S	Paymaster 2379
TX	12270	REF-0203-12270-S	AFD Rocket
TX	12271	REF-0203-12271-S	All-Tex Atlas

[1]Baldwin County, Alabama (AL); Tulare County, California (CA); Clarke County, Georgia (GA); Hockley County, Texas (TX).
[2]Conventional commercial cotton varieties

Table C-14. Roundup UltraMAX Herbicide Applications for 2002 Field Sample Production.

Site Code[1]	Material Treated with Product	Product Applied	Growth Stage	Application Date (mm/dd/yy)	Application Rate[2] (oz/A)
AL	12170	Roundup UltraMAX	3 node	06/04/02	40
			8 node	06/20/02	40
			Early bloom	07/16/02	40
CA	12170	Roundup UltraMAX	3 node	06/15/02	40
			8 node	06/27/02	40
			First flower	07/13/02	40
GA	12170	Roundup UltraMAX	2-4 node	06/30/02	40
			6-8 node	07/17/02	40
			First flower	08/01/02	40
TX	12170	Roundup UltraMAX	4-5 node	06/25/02	42
			8-10 node	07/08/02	40
			First flower	07/29/02	40

[1]Baldwin County, Alabama (AL); Tulare County, California (CA); Clarke County, Georgia (GA); Hockley County, Texas (TX).
[2]Product application rate. Roundup UltraMAX herbicide applied at 40 ounces per acre is equivalent to 1.125 lb ae/A of the acid, glyphosate.

Table C-15. Young Leaf and Over-Season Leaf Sampling from the 2002 Field Production.

Site Code[1]	Sample Type	Collection Date (mm/ddy/yy)	Crop Stage at Sampling	Sample Size (# of leaves)
AL	YL	05/30/02	1 node	16-30
	OSL-1	06/10/02	3-5 node[2]	14-25
	OSL-2	07/22/02	50% flower	25
	OSL-3	09/03/02	Cut-out[3]	25
CA	YL	06/10/02	1 node	30
	OSL-1	06/24/02	4 node	25
	OSL-2	07/16/02	50% flower	25
	OSL-3	09/09/02	Cut-out	25
GA	YL	06/19/02	1 node	24-30
	OSL-1	07/02/02	3-5 node	25
	OSL-2	08/12/02	50% flower	25
	OSL-3	08/28/02	Cut-out	25
TX	YL	06/19/02	2-3 nodes	1-20
	OSL-1	06/26/02	4-6 node	4-25
	OSL-2	08/09/02	50% flower	25
	OSL-3	08/28/02	Cut-out	25

Samples collected from MON 88913 and MON 88913(-) plots.
[1]Baldwin County, Alabama (AL); Tulare County, California (CA); Clarke County, Georgia (GA); Hockley County, Texas (TX).
[2]Plant growth stage estimated by days after planting.
[3]Cut-out generally refers to the stage in cotton when vegetative growth ceases.
YL = Young Leaf
OSL = Over-season leaf

Table C-16. Root Sampling from 2002 Field Production.

Site Code[1]	Collection Date (mm/dd/yy)	Crop Stage at Sampling	Sample Size (# of Roots)
AL	07/22/02	50% flower	3[2]
CA	07/17/02	50% flower	3
GA	08/13/02	50% flower	3
TX	08/07/02 and 08/09/02	50% flower	3[2]

Samples collected from MON 88913 and MON 88913(-) plots
[1]Baldwin County, Alabama (AL); Tulare County, California (CA); Clarke County, Georgia (GA); Hockley County, Texas (TX).
[2]Samples not collected from some MON 88913(-) plots due to limited plant stand.

Table C-17. Pollen Sampling from 2002 Field Production.

Site Code[1]	Collection Date (mm/dd/yy)[2]	Amount Collected (ml)
AL	07/22/02	~5
CA	07/22/02	~3
GA	08/12/02	2-3
TX	08/09/02	2-3

Samples collected from MON 88913 and MON 88913(-) plots.
[1]Baldwin County, Alabama (AL); Tulare County, California (CA); Clarke County, Georgia (GA); Hockley County, Texas (TX).
[2]Pollen samples were collected at the 50% flower growth stage.

C.4. Reproductive Tolerance and Floral Phenotypic Characteristics

Data were generated to determine the effects of over-the-top, sequential applications of glyphosate on MON 88913 on pollen viability, pollen availability, and floral morphology. The results are discussed in Section VII., and individual results and comparisons are presented below.

The Roundup agricultural herbicide label recommendation for a single over-the-top application in Roundup Ready cotton is equivalent to 0.76 lb ae/A applied no later than the four-leaf (node) stage of development. Additionally, the label stipulates that no more than two single over-the-top applications are to be made prior to the four-leaf (node) stage, spaced no less than 10 days and two nodes of incremental growth apart. Additionally, any single over-the-top application should not exceed 1 qt (0.76 lb ae) per acre. Over-the-top applications made after the four-leaf (node) stage of development may result in boll loss, delayed maturity and/or yield loss. The potential for reduced performance in Roundup Ready cotton following glyphosate applications outside of these restrictions has been attributed to low pollen grain viability (Chen and Hubmeier, 2001; Pline et al., 2002), reduced pollen availability, and failure to obtain successful pollination (Mery et al., 2002). Roundup Ready Flex cotton MON 88913 has been designed to enhance the reproductive tolerance to glyphosate when compared to that currently obtained with Roundup Ready cotton.

MON 88913, MON 88913(-), and Roundup Ready cotton were grown in a greenhouse. Roundup WeatherMAX herbicide was sequentially applied over the top of MON 88913 and Roundup Ready cotton at three different stages of growth: four-leaf (node), eight-leaf (node), and 12-leaf (node). No glyphosate applications were made to MON 88913(-). Pollen availability, pollen viability, stamen length (anther + filament), staminal column height, anther height (anther height as a percent of the pistil length), and pollen deposition were determined. Following the three applications of glyphosate, at 1.5 lb ae/A per application, the number of pollen grains attached to a stigmatic lobe of the glyphosate-treated MON 88913 was markedly increased over treated Roundup Ready cotton (Table C-18). No significant differences were detected in the number of pollen grains on treated MON 88913 compared to untreated MON 88913, untreated MON 88913(-), or untreated Roundup Ready cotton. No significant difference was detected in the number of pollen grains attached to a stigmatic lobe of untreated MON 88913 compared to untreated MON 88913(-).

Under the rate of glyphosate applied, the degree of anther dehiscence was markedly decreased in treated Roundup Ready cotton compared to treated MON 88913 (Table C-19). The degree of anther dehiscence in treated MON 88913 flowers was less than that of untreated MON 88913 and untreated Roundup Ready cotton. No differences were detected in anther dehiscence between treated MON 88913 and the untreated control, MON 88913(-). No significant differences in anther dehiscence were detected between untreated MON 88913 and untreated MON 88913(-) (Table C-19). Percent pollen viability was significantly greater in treated MON 88913 compared to treated Roundup Ready cotton, as evidenced by both Brewbaker and Kwack and Alexander's stain for pollen viability (Tables C-20 and C-21, respectively). There were no significant

differences detected in percent pollen viability between untreated MON 88913 and untreated MON 88913(-) when either staining method was used.

Stamen length (filament + anther) in treated MON 88913 was greater than that in treated Roundup Ready cotton. Stamen length was less in treated MON 88913 than in either untreated MON 88913 or untreated MON 88913(-) (Table C-22). There was no significant difference detected in stamen length between untreated MON 88913 and the untreated MON 88913(-). No difference was detected in the staminal column height between treated MON 88913 and treated Roundup Ready cotton. The staminal column height was reduced in treated MON 88913, compared to untreated MON 88913, and untreated MON 88913(-) (Table C-23). There was no difference detected in staminal column height between untreated MON 88913 and untreated MON 88913(-). Anther height, calculated as percent of pistil length, was greater for treated MON 88913 than for treated Roundup Ready cotton, but less than untreated MON 88913 (Table C-24). No difference was detected in anther height between treated MON 88913 and untreated MON 88913(-). Untreated MON 88913 anther height as a percent of pistil length was greater than untreated MON 88913(-) (Table C-24). This small percentage difference (4%) would convert to a relatively minor actual height difference and would have little biological meaning in terms of flower morphology or function. Distribution of pollen on the stigmatic surface of treated MON 88913 was greater than treated Roundup Ready cotton (Table C-25). The pollen deposition rating for treated MON 88913 was lower, however, than that for untreated MON 88913. There were no differences detected in pollen deposition between untreated MON 88913 and untreated MON 88913(-) (Table C-25).

MON 88913 demonstrated significantly increased reproductive tolerance and percent pollen viability compared to Roundup Ready cotton. Stamen length, anther height, and pollen deposition were also greater in MON 88913 compared to Roundup Ready cotton. For all parameters evaluated, except anther height as a percent of pistil length, there were no significant differences detected between untreated MON 88913 and untreated MON 88913(-). Untreated MON 88913 anther height as a percent of pistil length was greater than untreated MON 88913(-), but the small percentage difference (4%) would have little biological meaning in terms of flower morphology or function; this was corroborated by the field plant mapping data in the previous field phenotypic analyses. These data support the conclusion that MON 88913 possesses a significantly enhanced margin of crop safety for glyphosate-based herbicides.

Table C-18. Number of Pollen Grains on Stigmatic Lobe.

Materials	Glyphosate Treatment[1]	Pollen grains attached to one stigmatic lobe[2] (#)
MON 88913	Treated	131ab
MON 88913	Untreated	139ab
MON 88913(-)	Untreated	142a
Roundup Ready cotton	Treated	7c
Roundup Ready cotton	Untreated	116b

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean number of pollen grains attached to the stigmatic lobe with greatest number of pollen grains. Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-19. Anther Dehiscence.

Materials	Glyphosate Treatment[1]	Anther rating for dehiscence[2]
MON 88913	Treated	3.1b
MON 88913	Untreated	3.7a
MON 88913(-)	Untreated	3.5ab
Roundup Ready cotton	Treated	0.1c
Roundup Ready cotton	Untreated	3.8a

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean anther dehiscence ratings where 0 = 0% dehisced; 1 = 25% dehisced; 2 = 50% dehisced; 3 = 75% dehisced; 4 = 100% dehisced (open). Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-20. Pollen Viability with Brewbaker and Kwack Staining Method.

Materials	Glyphosate Treatment[1]	Percent pollen viability[2] (%)
MON 88913	Treated	74b
MON 88913	Untreated	85a
MON 88913(-)	Untreated	90a
Roundup Ready cotton	Treated	6c
Roundup Ready cotton	Untreated	91a

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean percent viable pollen. Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-21. Pollen Viability with Alexander Staining Method.

Materials	Glyphosate Treatment[1]	Percent pollen viability[2] (%)
MON 88913	Treated	84b
MON 88913	Untreated	95a
MON 88913(-)	Untreated	95a
Roundup Ready cotton	Treated	6c
Roundup Ready cotton	Untreated	94a

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean percent viable pollen. Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-22. Stamen Length.

Materials	Glyphosate Treatment[1]	Stamen length[2] (mm)
MON 88913	Treated	5.5b
MON 88913	Untreated	5.9a
MON 88913(-)	Untreated	5.9a
Roundup Ready cotton	Treated	4.4c
Roundup Ready cotton	Untreated	6.1a

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean stamen length = anther + filament. Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-23. Staminal Column Height.

Materials	Glyphosate Treatment[1]	Staminal column[2] (mm)
MON 88913	Treated	10.1c
MON 88913	Untreated	11.6b
MON 88913(-)	Untreated	11.9b
Roundup Ready cotton	Treated	10.2c
Roundup Ready cotton	Untreated	12.7a

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean staminal column length (including ovary). Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-24. Anther Height.

Materials	Glyphosate Treatment[1]	Anther height[2] (% pistil length)
MON 88913	Treated	94b
MON 88913	Untreated	100a
MON 88913(-)	Untreated	96b
Roundup Ready cotton	Treated	78c
Roundup Ready cotton	Untreated	94b

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean of the uppermost anther height as a percent of pistil length; anther height as % of pistil length calculated from the raw data = 100 – ({[pistil length – uppermost anther height] / pistil length} x 100). Numbers followed by the same letter were not significantly different at the 5% level of significance.

Table C-25. Pollen Deposition.

Materials	Glyphosate Treatment[1]	Pollen deposition rating[2]
MON 88913	Treated	2.1b
MON 88913	Untreated	2.6a
MON 88913(-)	Untreated	2.6a
Roundup Ready cotton	Treated	0.2d
Roundup Ready cotton	Untreated	1.2c

[1] 1.5 lb ae/A glyphosate application at 4-, 8-, and 12-leaf (node) stages.
[2] Mean pollen deposition rating where 0 = no pollen attached; 1 = distribution over lower 2/3 of stigma; 2 = distribution over upper 2/3 of stigma; 3 = even distribution over entire stigma. Numbers followed by the same letter were not significantly different at the 5% level of significance.

Appendix D: Characterization of the CP4 EPSPS Protein in MON 88913

Appendix D: Characterization of the CP4 EPSPS Protein in MON 88913

The physicochemical properties of the CP4 EPSPS protein isolated from MON 88913 were determined and the equivalence of the MON 88913-produced protein to the previously characterized *E. coli*-produced CP4 EPSPS protein was assessed. The methods used in these analyses are described in Appendix B.

N-terminal Sequence Analysis

The results of the N-terminal sequence analysis of the plant-produced CP4 EPSPS protein are summarized in Table D-1. The experimentally determined N-terminal sequence for the plant-produced CP4 EPSPS isolated from MON 88913 confirmed the expected amino acid sequence. Three sequences, all of which are consistent with the N-terminus of the CP4 EPSPS protein, were observed in the CP4 EPSPS protein isolated from MON 88913 seed. The first sequence originates at residue four, glycine, and the other two sequences start at residues two, and six (leucine, and serine, respectively). The observation of a staggered N-terminal sequence for the plant-produced CP4 EPSPS protein has previously been reported for cotton (Harrison et al., 1996) and soybean (Harrison et al., 1996). Such a finding is not uncommon because the initiator methionine is normally removed from proteins in eukaryotic organisms by an endogenous methionine aminopeptidase (Arfin and Bradshaw, 1988) and the loss of several N-terminal amino acid residues may be due to protease action when plant cells are homogenized. Despite the staggered N-terminus, the sequence data confirm that the ~43 kDa protein isolated from the seed of MON 88913 is the CP4 EPSPS protein and that this sequence is consistent with the N-terminal sequence of the *E. coli*-produced CP4 EPSPS reference standard.

Table D-1. N-terminal Amino Acid Sequence Analysis of the CP4 EPSPS Protein Purified from MON 88913.

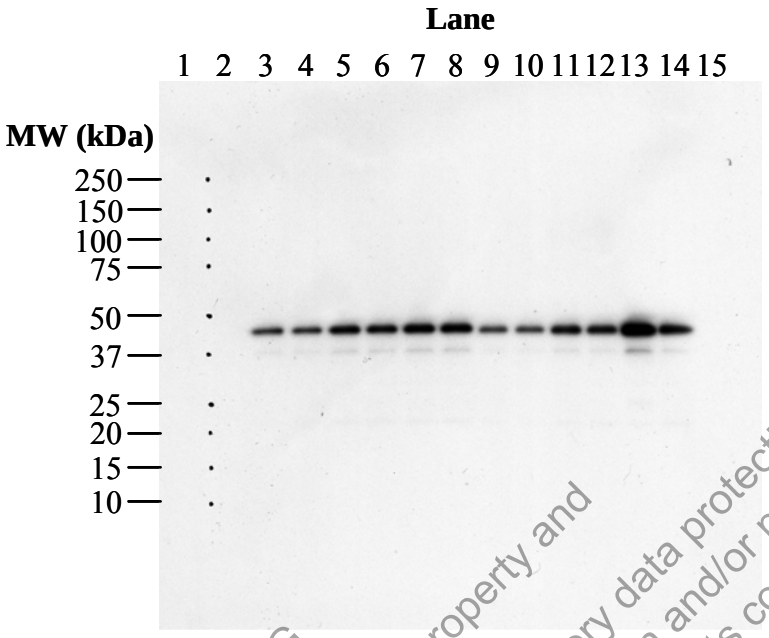
The predicted amino acid sequence (residues 1-20 of 455) of the plant-produced CP4 EPSPS protein was deduced from the coding region of the full-length *cp4 epsps* coding sequences present in MON 88913. Three sequences were observed (1, 2, and 3) from N-terminal sequencing of the ~43 kDa band, all of which are consistent with plant-produced CP4 EPSPS protein. For all sequences, undesignated amino acid assignments are shown as an "X," tentative assignments are shown in brackets ( ) and amino acids are assigned using the single letter amino acid code[a].

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Predicted	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	G	L	S	G
Observed-1				G	A	S	X	R	P	A	T	A	R	K	S	X	G	(L)		
Observed-2						S	X	R	P	A	T	A	X	K	S	S	G	L	S	(G)
Observed-3		L	H	G	A	X	X	R	X	A	X	X	X	X	S	X				

[a]The single letter IUPAC-IUB amino acid code is **A**, alanine; **G**, glycine; **H**, histidine; **K**, lysine; **L**, leucine; **M**, methionine; **P**, proline; **R**, arginine; **S**, serine; and **T**, threonine.

Immunoblot Analysis – Immunoreactivity.

Immunoblot analysis was performed using goat anti-CP4 EPSPS serum, which was produced using an *E. coli*-produced CP4 EPSPS protein as the antigen. The plant-produced CP4 EPSPS and reference standard were loaded in duplicate at 1, 2, and 3 ng CP4 EPSPS protein per lane. As expected, the immunoreactive signal increased with increased levels of the CP4 EPSPS protein (Figure D-1; Table D-2). However, there was a difference in the immunoreactive signals among duplicates for the plant-produced CP4 EPSPS loaded at 3 ng. This observed difference was likely because of an error in loading the duplicate sample in lane # 13. Thus, the densitometric value for this lane was excluded from the average calculation of immunoreactivity of plant-produced CP4 EPSPS protein. Also visible are lower molecular weight immunoreactive bands in lanes 3-8 and 9-14 that migrate at approximately 23 kDa and 37 kDa. The lower molecular weight immunoreactive bands, visible with increased levels of the loaded proteins, may have been formed by proteolytic degradation of CP4 EPSPS protein during the protein extraction process. Furthermore, the western blot analysis showed that the CP4 EPSPS protein isolated from MON 88913 bound equivalent amounts of goat anti-CP4 EPSPS serum (overall average percent difference of ≤ 10%) to the *E. coli*-produced CP4 EPSPS reference standard. The observed similarity in protein mobility and immunoreactivity for the plant- and *E. coli*-produced CP4 EPSPS proteins demonstrates that the plant-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS reference standard.



Lane	Sample	Amount (ng)
1	Blank lane containing 10 µl Laemmli sample buffer	—
2	MW Markers	—
3	*E. coli*-produced CP4 EPSPS reference standard	1
4	*E. coli*-produced CP4 EPSPS reference standard	1
5	*E. coli*-produced CP4 EPSPS reference standard	2
6	*E. coli*-produced CP4 EPSPS reference standard	2
7	*E. coli*-produced CP4 EPSPS reference standard	3
8	*E. coli*-produced CP4 EPSPS reference standard	3
9	Plant-produced CP4 EPSPS protein from MON 88913	1
10	Plant-produced CP4 EPSPS protein from MON 88913	1
11	Plant-produced CP4 EPSPS protein from MON 88913	2
12	Plant-produced CP4 EPSPS protein from MON 88913	2
13	Plant-produced CP4 EPSPS protein from MON 88913	3
14	Plant-produced CP4 EPSPS protein from MON 88913	3
15	Blank lane containing 10 µl Laemmli sample buffer	—

Figure D-1. Immunoblot Analysis of the CP4 EPSPS Protein Isolated from MON 88913.

Samples of plant-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS reference standard were separated by 4→20% SDS-PAGE, electrotransferred to a PVDF membrane and detected using CP4 EPSPS polyclonal antiserum followed by development using the ECL system (15 sec exposure shown). Amount refers to CP4 EPSPS protein (corrected for purity) loaded per lane. Approximate molecular weights (kDa) correspond to the markers loaded in Lane 2.

Table D-2. Summary of the Densitometric Analysis of the Immunoblot of the *E. coli*- and Plant-Produced CP4 EPSPS Proteins.

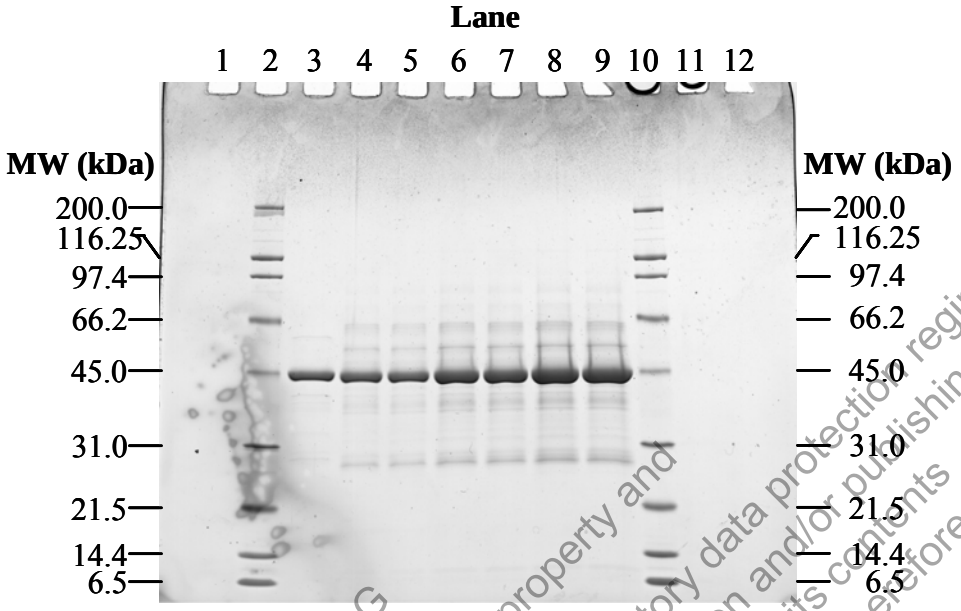
Sample	Lane	Load (ng)	Replicate	Contour quantity	Average of Replicates	Percent difference between replicate averages
Test Protein	9	1	1	2.622	2.74	4.20
Test Protein	10	1	2	2.851		
Reference Protein	3	1	1	2.891	2.86	
Reference Protein	4	1	2	2.822		
Test Protein	11	2	1	5.684	5.72	6.32
Test Protein	12	2	2	5.747		
Reference Protein	5	2	1	5.475	5.38	
Reference Protein	6	2	2	5.277		
Test Protein	13	3	1	14.83[a]	6.72	11.11
Test Protein	14	3	2	6.716		
Reference Protein	7	3	1	7.729	7.56	
Reference Protein	8	3	2	7.388		
					Average Percent Difference→	7.21

[a] Although lanes 13 and 14 were loaded with duplicate loadings of 3 ng protein, lane 13 showed a higher signal than lane 14. Comparing the signal from the duplicate and the trend in increasing signal with increasing protein loading, it was concluded that the higher signal observed in lane 13 was likely because of an error in the loading of the gel and, thus, this value was excluded from the average calculation. However, it should be noted that even when lane #13 was included in the calculation of Percentage Difference, the average difference was less than 20%, which was considered the threshold for equivalent immunoreactivity.

Contour quantities of each band in the test and reference proteins were determined by densitometric analysis of the X-ray film depicted in Figure D-1. For each pair of replicates, the average contour quantity was calculated and then the average contour quantities for equal loadings of the test and reference proteins were compared. The overall average difference in contour quantities for 1, 2, and 3 ng loadings of the test and reference proteins was 7.21%.

Molecular Weight and Purity Determination.

The plant-produced CP4 EPSPS protein was separated using SDS-PAGE and stained with Brilliant Blue G-Colloidal stain (Figure D-2). The purity and molecular weight of the plant-produced CP4 EPSPS protein were estimated using densitometric analysis (Table D-3). The predominant band in the plant-purified sample had an average molecular weight of 43.1 kDa. Because this protein migrated with a near identical molecular weight as that of the *E. coli*-produced CP4 EPSPS reference standard (43.8 kDa), the plant-produced CP4 EPSPS protein was concluded to have the same MW as the *E. coli*-produced CP4 EPSPS protein. The average purity of the plant-produced CP4 EPSPS protein was estimated to be 81%.



Lane	Sample	Amount (µg)
1	Blank lane containing 10 µl Laemmli sample buffer	—
2	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
3	*E. coli*-produced CP4 EPSPS reference standard	1
4	Plant-produced CP4 EPSPS protein from MON 88913	1
5	Plant-produced CP4 EPSPS protein from MON 88913	1
6	Plant-produced CP4 EPSPS protein from MON 88913	2
7	Plant-produced CP4 EPSPS protein from MON 88913	2
8	Plant-produced CP4 EPSPS protein from MON 88913	3
9	Plant-produced CP4 EPSPS protein from MON 88913	3
10	MW Markers (Bio-Rad, Cat #: 161-0317)	0.5 µg/band
11	Blank lane containing 10 µl Laemmli sample buffer	—
12	Empty lane (nothing loaded into the well)	—

Figure D-2. SDS-PAGE Purity and Molecular Weight Analysis of the CP4 EPSPS Protein Isolated from MON 88913.

Samples of the plant-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS reference standard were loaded as indicated on a 4→20% polyacrylamide gel. Amount refers to total protein loaded per lane. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

Table D-3. Protein Molecular Weight and Purity Estimation of the CP4 EPSPS Protein Isolated from MON 88913.

1 µg Load (Figure D-2, Lanes 4 and 5)				2 µg Load (Figure D-2, Lanes 6 and 7)				3 µg Load (Figure D-2, Lanes 8 and 9)				Average Value	
Replicate 1		**Replicate 2**		**Replicate 1**		**Replicate 2**		**Replicate 1**		**Replicate 2**			
MW	**RQTY**	**MW**	**RQTY**	**MW**	**RQTY**	**MW**	**RQTY**	**MW**	**RQTY**	**MW**	**RQTY**	**MW**	**RQTY**
—	—	—	—	—	—	—	—	86.95	0.6	86.81	0.5	—	—
—	—	—	—	—	—	—	—	75.75	0.4	75.34	0.3	—	—
63.65	1.6	63.48	1.4	63.91	1.5	63.65	2.0	64.48	1.5	64.46	1.2	—	—
61.25	1.2	62.27	1.3	62.55	1.4	62.60	1.5	63.27	2.1	62.95	1.8	—	—
53.41	3.2	53.42	2.9	53.94	3.7	53.99	3.7	54.46	3.5	54.33	3.5	—	—
43.32	**83.5**	**43.33**	**83.8**	**42.92**	**79.8**	**43.04**	**80.1**	**42.94**	**78.6**	**42.96**	**78.9**	**43.1**	**81**
39.95	0.8	40.03	0.6	40.23	0.8	40.34	0.6	40.51	0.6	40.33	1.0	—	—
38.13	2.4	38.20	2.2	38.58	3.2	38.68	2.9	38.90	3.4	38.85	3.2	—	—
36.79	0.7	36.86	0.9	37.11	1.0	37.27	1.0	37.48	1.1	37.49	1.0	—	—
—	—	—	—	32.24	0.3	32.38	0.4	32.50	0.4	32.45	0.3	—	—
29.24	1.3	29.19	1.4	29.69	1.5	29.79	1.3	29.92	1.7	29.92	1.6	—	—
27.79	5.2	27.95	5.4	28.22	6.2	28.36	5.7	28.54	5.6	28.44	6.2	—	—
—	—	—	—	9.43	0.5	9.70	0.8	10.28	0.5	10.09	0.6	—	—

Relative percent quantities (RQTY) of each visible band in the test protein (isolated from MON 88913) were derived from densitometric analysis of the SDS polyacrylamide gel shown in Figure D-2, Lanes 4 to 9. The test protein MW (kDa) were calculated from the molecular weight markers (Figure D-2, Lanes 2 and 10) using the manufacturer's supplied molecular weight values. The average molecular weight and purity of the plant-produced CP4 EPSPS protein were determined to be ~43.1 kDa and 81% (shown in bold values), respectively. The row with the numbers in bold type corresponds to the major band on the SDS polyacrylamide gel which is the CP4 EPSPS protein.

Conclusions for MON 88913 CP4 EPSPS Protein Characterization

A panel of analytical tests, some using the *E. coli*-produced CP4 EPSPS protein as a reference standard, was used to characterize the plant-produced CP4 EPSPS protein. The identity of the plant-produced protein was confirmed using data from immunoblot analysis and N-terminal sequence analysis. On the basis of western blot analysis, the electrophoretic mobility and immunoreactive properties of the plant-produced CP4 EPSPS protein were demonstrated to be comparable to those of the *E. coli*-produced CP4 EPSPS reference standard. The N-terminus of the major protein band contained in the plant-produced CP4 EPSPS protein preparation was sequenced using automated Edman degradation, and the amino acid sequence was found to be consistent with the predicted sequence of amino acids translated from the *cp4 epsps* coding sequence within MON 88913. The molecular weight of the plant-produced CP4 EPSPS protein was estimated using SDS-PAGE and densitometric analysis. The predominant band in the plant-purified sample had an average molecular weight of 43.1 kDa, and migrated with a near identical molecular weight as that of the *E. coli*-produced CP4 EPSPS reference standard (43.8 kDa).

Collectively, these data establish the physicochemical properties of the CP4 EPSPS protein isolated from MON 88913 and establish its equivalence to the *E. coli*-produced CP4 EPSPS protein used in studies to determine the safety of the CP4 EPSPS protein.

Appendix E: Compositional Analysis Tables

Appendix E: Compositional Analysis Tables

In order to assess the composition of MON 88913 cottonseed, a compositional analysis was conducted of delinted cottonseed grown under replicated field conditions in the U.S. at four sites. As in the field phenotypic assessment, MON 88913 was compared MON 88913(-), which has background genetics representative of the test material but does produce the CP4 EPSPS protein. Sixteen commercial conventional cotton varieties produced in the same field trial alongside MON 88913 and MON 88913(-) were also analyzed as references. A summary of data where statistical differences were observed was presented in the main body of the text. The detailed compositional values are presented in the tables in this Appendix, as well as literature values for cottonseed compositional analytes.

Table E-1. Statistical Summary of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical[1] Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Amino Acid (% Total AA)						
Alanine	4.28 ± 0.056	4.30 ± 0.056	-0.013 ± 0.030	-0.11,0.081	0.691	(4.08 - 4.46)
	(4.09 - 4.51)	(4.15 - 4.46)	(-0.27 - 0.24)			[4.01,4.58]
Arginine	11.78 ± 0.17	11.77 ± 0.17	0.0033 ± 0.12	-0.39,0.40	0.980	(11.08 - 12.77)
	(11.19 - 12.25)	(11.11 - 12.27)	(-0.81 - 0.99)			[10.57,12.96]
Aspartic Acid	9.82 ± 0.064	9.80 ± 0.064	0.020 ± 0.031	-0.080,0.12	0.567	(9.70 - 10.38)
	(9.59 - 10.08)	(9.59 - 9.99)	(-0.13 - 0.29)			[9.48,10.35]
Cystine	1.89 ± 0.042	1.92 ± 0.042	-0.035 ± 0.029	-0.097,0.027	0.243	(1.62 - 2.35)
	(1.69 - 2.10)	(1.76 - 2.10)	(-0.25 - 0.16)			[1.60,2.14]
Glutamic Acid	21.66 ± 0.13	21.55 ± 0.13	0.11 ± 0.096	-0.085,0.31	0.253	(20.92 - 22.18)
	(21.08 - 22.14)	(21.10 - 21.96)	(-0.63 - 1.03)			[20.88,22.49]
Glycine	4.42 ± 0.029	4.45 ± 0.029	-0.025 ± 0.018	-0.062,0.012	0.171	(4.29 - 4.66)
	(4.33 - 4.56)	(4.33 - 4.64)	(-0.24 - 0.13)			[4.21,4.64]

[1]Means in the table are least square means from SAS. Cottonseed produced under field conditions in 2002 from Baldwin County, Alabama; Tulare County, California; Clarke County, Georgia; Hockley County, Texas.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Amino Acid (% Total AA)						
Histidine	3.15 ± 0.0079	3.14 ± 0.0079	0.0059 ± 0.011	-0.022,0.033	0.619	(3.01 - 3.22)
	(3.09 - 3.21)	(3.11 - 3.20)	(-0.070 - 0.10)			[3.04,3.23]
Isoleucine	3.43 ± 0.020	3.43 ± 0.020	-0.0040 ± 0.026	-0.086,0.078	0.887	(3.19 - 3.59)
	(3.31 - 3.54)	(3.34 - 3.56)	(-0.25 - 0.12)			[3.13,3.65]
Leucine	6.31 ± 0.048	6.27 ± 0.048	0.046 ± 0.026	-0.036,0.13	0.169	(6.03 - 6.48)
	(6.14 - 6.52)	(6.10 - 6.48)	(-0.20 - 0.20)			[5.84,6.66]
Lysine	4.99 ± 0.052	5.09 ± 0.052	-0.11 ± 0.053	-0.22,0.0020	0.053	(4.72 - 5.38)
	(4.77 - 5.23)	(4.89 - 5.48)	(-0.48 - 0.30)			[4.53,5.43]
Methionine	1.65 ± 0.040	1.69 ± 0.040	-0.042 ± 0.043	-0.18,0.094	0.397	(1.27 - 1.94)
	(1.47 - 1.90)	(1.49 - 1.95)	(-0.34 - 0.22)			[1.30,1.93]
Phenylalanine	5.64 ± 0.014	5.60 ± 0.014	0.044 ± 0.019	0.0042,0.083	0.031	(5.44 - 5.82)
	(5.53 - 5.75)	(5.45 - 5.72)	(-0.19 - 0.21)			[5.43,5.82]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Amino Acid (% Total AA)						
Proline	4.17 ± 0.045	4.16 ± 0.045	0.010 ± 0.028	-0.079,0.099	0.739	(3.97 - 4.49)
	(3.92 - 4.39)	(3.93 - 4.25)	(-0.18 - 0.20)			[3.91,4.43]
Serine	4.88 ± 0.096	4.90 ± 0.096	-0.017 ± 0.054	-0.19,0.15	0.773	(4.53 - 5.31)
	(4.35 - 5.32)	(4.65 - 5.32)	(-0.48 - 0.50)			[4.55,5.42]
Threonine	3.19 ± 0.094	3.20 ± 0.094	-0.0082 ± 0.067	-0.22,0.21	0.910	(2.67 - 3.50)
	(2.61 - 3.49)	(2.70 - 3.45)	(-0.49 - 0.48)			[2.73,3.74]
Tryptophan	1.10 ± 0.012	1.14 ± 0.012	-0.039 ± 0.016	-0.074,-0.0044	0.029	(0.97 - 1.31)
	(1.03 - 1.23)	(1.09 - 1.25)	(-0.14 - 0.089)			[0.94,1.26]
Tyrosine	2.79 ± 0.033	2.78 ± 0.033	0.017 ± 0.028	-0.071,0.11	0.576	(2.63 - 2.93)
	(2.70 - 2.90)	(2.62 - 2.89)	(-0.085 - 0.18)			[2.61,3.00]
Valine	4.84 ± 0.028	4.81 ± 0.028	0.032 ± 0.024	-0.019,0.084	0.202	(4.57 - 5.02)
	(4.68 - 5.00)	(4.68 - 4.96)	(-0.12 - 0.22)			[4.48,5.02]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Fatty Acid (% Total FA)						
14:0 Myristic	0.76 ± 0.040	0.75 ± 0.040	0.016 ± 0.019	-0.044,0.077	0.458	(0.64 - 1.03)
	(0.66 - 0.90)	(0.65 - 0.90)	(-0.092 - 0.20)			[0.44,1.14]
16:0 Palmitic	23.55 ± 0.40	23.09 ± 0.40	0.46 ± 0.19	-0.13,1.05	0.089	(21.47 - 25.36)
	(22.09 - 24.69)	(21.26 - 24.17)	(-0.41 - 1.67)			[20.76,26.19]
16:1 Palmitoleic	0.54 ± 0.0066	0.53 ± 0.0066	0.0098 ± 0.0086	-0.0079,0.027	0.265	(0.46 - 0.77)
	(0.51 - 0.59)	(0.50 - 0.59)	(-0.048 - 0.090)			[0.37,0.80]
18:0 Stearic	2.64 ± 0.073	2.65 ± 0.073	-0.0096 ± 0.041	-0.14,0.12	0.830	(2.38 - 3.03)
	(2.32 - 2.85)	(2.33 - 2.94)	(-0.28 - 0.17)			[2.18,3.17]
18:1 Oleic	18.61 ± 0.75	20.94 ± 0.75	-2.33 ± 0.27	-3.18,-1.48	0.003	(13.29 - 18.60)
	(16.35 - 20.72)	(18.34 - 23.29)	(-4.07 - -0.56)			[10.59,21.29]
18:2 Linoleic	52.36 ± 0.76	50.42 ± 0.76	1.94 ± 0.28	1.34,2.53	<0.001	(51.51 - 59.40)
	(49.66 - 54.32)	(47.89 - 53.27)	(-0.36 - 3.40)			[48.89,61.11]
18:3 Gamma Linolenic	0.12 ± 0.023	0.13 ± 0.023	-0.0059 ± 0.031	-0.10,0.092	0.860	(0.043 - 0.23)
	(0.045 - 0.28)	(0.049 - 0.20)	(-0.13 - 0.23)			[0,0.24]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Fatty Acid (% Total FA)						
18:3 Linolenic	0.18 ± 0.025	0.17 ± 0.025	0.0044 ± 0.0076	-0.020,0.029	0.602	(0.11 - 0.27)
	(0.11 - 0.26)	(0.12 - 0.24)	(-0.031 - 0.042)			[0.031,0.31]
20:0 Arachidic	0.27 ± 0.0057	0.28 ± 0.0057	-0.00064 ± 0.0054	-0.018,0.016	0.913	(0.22 - 0.33)
	(0.25 - 0.31)	(0.24 - 0.30)	(-0.031 - 0.043)			[0.21,0.34]
22:0 Behenic	0.15 ± 0.0048	0.15 ± 0.0048	0.00095 ± 0.0038	-0.0071,0.0090	0.804	(0.12 - 0.18)
	(0.13 - 0.17)	(0.12 - 0.17)	(-0.024 - 0.023)			[0.099,0.19]
Dihydrosterculic	0.15 ± 0.0081	0.17 ± 0.0081	-0.021 ± 0.0075	-0.045,0.0028	0.067	(0.075 - 0.24)
	(0.12 - 0.18)	(0.10 - 0.21)	(-0.062 - 0.031)			[0.056,0.25]
Malvalic	0.36 ± 0.040	0.39 ± 0.040	-0.035 ± 0.029	-0.13,0.056	0.310	(0.23 - 0.56)
	(0.24 - 0.56)	(0.23 - 0.55)	(-0.22 - 0.13)			[0.16,0.58]
Sterculic	0.31 ± 0.025	0.33 ± 0.025	-0.024 ± 0.016	-0.057,0.0097	0.157	(0.19 - 0.41)
	(0.24 - 0.41)	(0.21 - 0.44)	(-0.17 - 0.094)			[0.18,0.40]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Fiber (% dwt)						
Acid Detergent Fiber	31.31 ± 0.50	30.78 ± 0.50	0.53 ± 0.64	-0.77,1.83	0.409	(26.32 - 38.97)
	(27.72 - 34.98)	(28.08 - 34.42)	(-6.70 - 5.74)			[25.48,38.48]
Crude Fiber	17.76 ± 0.68	17.97 ± 0.68	-0.21 ± 0.38	-1.42,1.00	0.616	(15.96 - 23.10)
	(14.96 - 20.41)	(16.04 - 20.39)	(-2.25 - 1.94)			[13.34,24.17]
Neutral Detergent Fiber	42.26 ± 1.07	42.56 ± 1.07	-0.29 ± 0.94	-2.22,1.63	0.757	(38.49 - 51.84)
	(33.91 - 47.36)	(38.00 - 46.92)	(-6.11 - 9.36)			[34.51,53.25]
Total Dietary Fiber	40.23 ± 0.53	39.60 ± 0.53	0.63 ± 0.48	-0.36,1.61	0.202	(36.47 - 47.54)
	(37.85 - 43.17)	(36.55 - 43.27)	(-4.90 - 3.03)			[36.13,48.96]
Mineral						
Calcium (% dwt)	0.16 ± 0.012	0.16 ± 0.012	-0.0024 ± 0.0063	-0.022,0.017	0.722	(0.10 - 0.19)
	(0.13 - 0.19)	(0.11 - 0.19)	(-0.022 - 0.020)			[0.074,0.22]
Copper (mg/kg dwt)	6.72 ± 0.61	6.54 ± 0.61	0.18 ± 0.20	-0.45,0.80	0.436	(4.92 - 12.47)
	(5.15 - 8.51)	(4.53 - 9.47)	(-1.83 - 1.06)			[2.01,12.94]
Iron (mg/kg dwt)	52.65 ± 1.68	52.20 ± 1.68	0.45 ± 1.42	-2.45,3.36	0.751	(36.71 - 67.75)
	(41.27 - 58.87)	(46.77 - 62.47)	(-21.20 - 8.45)			[33.44,68.99]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Mineral						
Magnesium (% dwt)	0.41 ± 0.011	0.42 ± 0.011	-0.0040 ± 0.0089	-0.032,0.024	0.682	(0.35 - 0.47)
	(0.38 - 0.45)	(0.37 - 0.46)	(-0.062 - 0.050)			[0.31,0.51]
Manganese (mg/kg dwt)	15.34 ± 1.29	14.64 ± 1.29	0.70 ± 0.29	0.096,1.30	0.024	(10.68 - 21.96)
	(12.37 - 19.98)	(11.91 - 18.23)	(-2.25 - 2.64)			[4.69,26.45]
Phosphorus (% dwt)	0.68 ± 0.052	0.70 ± 0.052	-0.013 ± 0.028	-0.10,0.075	0.671	(0.48 - 0.99)
	(0.54 - 0.82)	(0.53 - 0.93)	(-0.17 - 0.11)			[0.31,1.08]
Potassium (% dwt)	1.21 ± 0.030	1.23 ± 0.030	-0.014 ± 0.018	-0.073,0.044	0.488	(1.07 - 1.39)
	(1.12 - 1.34)	(1.12 - 1.43)	(-0.24 - 0.083)			[0.96,1.46]
Sodium (% dwt)	0.062 ± 0.015	0.068 ± 0.015	-0.0062 ± 0.019	-0.068,0.055	0.767	(0.032 - 0.14)
	(0.027 - 0.12)	(0.033 - 0.11)	(-0.075 - 0.034)			[0,0.17]
Zinc (mg/kg dwt)	40.87 ± 3.72	39.42 ± 3.72	1.45 ± 0.99	-0.58,3.47	0.155	(30.11 - 59.51)
	(29.30 - 52.16)	(27.60 - 52.16)	(-11.11 - 7.50)			[17.12,58.50]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Proximate						
Ash (% dwt)	4.33 ± 0.17	4.37 ± 0.17	-0.036 ± 0.084	-0.30,0.23	0.697	(3.76 - 5.34)
	(3.94 - 4.81)	(3.76 - 5.19)	(-0.82 - 0.39)			[2.96,5.62]
Calories (Kcal/100g dwt)	460.31 ± 5.33	455.51 ± 5.33	4.80 ± 3.80	-2.99,12.60	0.216	(407.45 - 471.46)
	(424.36 - 481.93)	(415.74 - 475.23)	(-34.58 - 36.83)			[409.12,496.45]
Carbohydrates (% dwt)	44.74 ± 0.49	45.57 ± 0.49	-0.83 ± 0.60	-2.73,1.08	0.260	(40.06 - 52.01)
	(42.61 - 47.67)	(42.07 - 49.32)	(-5.22 - 3.76)			[38.23,56.70]
Moisture (% fwt)	6.39 ± 0.26	6.22 ± 0.26	0.17 ± 0.063	0.038,0.30	0.013	(5.06 - 6.49)
	(5.65 - 7.34)	(5.32 - 7.12)	(-0.40 - 0.70)			[4.51,7.21]
Protein (% dwt)	28.23 ± 0.60	27.41 ± 0.60	0.82 ± 0.59	-0.39,2.03	0.175	(21.48 - 32.03)
	(24.08 - 31.13)	(21.64 - 29.53)	(-5.45 - 6.35)			[20.19,32.70]
Total Fat (% dwt)	22.70 ± 0.52	22.66 ± 0.52	0.046 ± 0.45	-1.38,1.48	0.925	(17.60 - 27.29)
	(21.00 - 25.25)	(19.99 - 24.82)	(-1.50 - 1.91)			[15.16,28.44]

Table E-1 (Continued). Statistical Summary[1] of Combined Site Cottonseed Amino Acid, Fatty Acid, Fiber, Mineral, Proximate, Vitamin and Gossypol Content for MON 88913 versus MON 88913(-).

			Difference [MON 88913 minus MON 88913(-)]			
Analytical Component	**MON 88913 Mean ± S.E. (Range)**	**MON 88913(-) Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower, Upper)**	***p-Value***	**Commercial (Range) [99% Tolerance Interval[2]]**
Vitamin						
Vitamin E (mg/kg dwt)	150.85 ± 14.02	148.79 ± 14.02	2.06 ± 1.93	-4.07,8.19	0.363	(70.79 - 197.22)
	(103.60 - 179.33)	(107.81 - 182.23)	(-6.89 - 14.75)			[9.30,263.66]
Gossypol						
Free Gossypol (% dwt)	0.65 ± 0.032	0.68 ± 0.032	-0.029 ± 0.036	-0.14,0.086	0.480	(0.53 - 1.05)
	(0.51 - 0.77)	(0.51 - 0.86)	(-0.19 - 0.16)			[0.43,1.06]
Total Gossypol (% dwt)	0.81 ± 0.034	0.82 ± 0.034	-0.0050 ± 0.018	-0.062,0.052	0.799	(0.78 - 1.19)
	(0.70 - 0.91)	(0.69 - 0.96)	(-0.16 - 0.099)			[0.61,1.25]

Table E-2. Literature Values for Cottonseed Compositional Analytes.

Component	Literature Ranges [a]
Proximates, Fibers (% dwt)	
Protein	21.2 [1] – 29.5 [2]
Fat	16.9 [3] – 26.8 [2]
Ash	3.8 [3] – 4.5 [4]
Moisture	5.4 [2] – 10.1 [2]
Carbohydrates	Not Available
Calories (kcal/100g)	Not Available
Acid Detergent Fiber	29.0 [5] – 40.1 [6]
Crude Fiber	20.8 [5]
Neutral Detergent Fiber	48.7 [3] – 50.3 [6]
Total Dietary Fiber	Not Available
Amino Acids (% Total AA)	
Alanine	3.6 [1] – 4.2 [1]
Arginine	10.9 [1] – 13.2 [1]
Aspartic Acid	8.8 [1] – 9.5 [1]
Cystine	1.76 [6] – 3.4 [1]
Glutamic Acid	19.9 [1] – 22.4 [1]
Glycine	3.7 [1] – 4.6 [1]
Histidine	2.6 [1] – 3.11 [6]
Isoleucine	2.8 [1] – 3.4 [1]
Leucine	5.3 [1] – 6.1 [1]
Lysine	4.2 [1] – 4.6 [1]
Methionine	1.2 [1] – 1.8 [1]
Phenylalanine	5.0 [1] – 6.2 [1]
Proline	3.1 [1] – 4.0 [1]
Serine	3.9 [1] – 4.4 [1]
Threonine	2.8 [1] – 3.46 [6]
Tryptophan	1.0 [1] – 1.4 [1]
Tyrosine	1.6 [1] – 3.3 [1]
Valine	4.1 [1] – 4.8 [1]

[a]Ranges include literature values for conventional cotton and for both glanded and glandless cotton. [1] Lawhon et al., 1977 (amino acids as g/16gN defatted flour); [2] Cherry et al., 1978 (fatty acids as % oil); [3] Belyea et al., 1989; [4] Cherry and Leffler, 1984; [5] NRC, 1982 (fuzzy seed; [6] NRC, 2001 (fuzzy seed, amino acids as % protein); [7] Cherry, 1983 (fatty acids as % lipid, 20:0 arachidic acid as % phospholipids in oil); [8] Shenstone and Vickery, 1961 (fatty acids as % oil); [9] Basset et al., 1970; [10] Cherry et al., 1986; [11] Smith and Creelman, 2001 (vitamin E as ppm fwt).

Table E-2 (Continued). Literature Values for Cottonseed Compositional Analytes.

Component	Literature Ranges [b]
Fatty Acids (% Total FA)	
14:0 Myristic	0.56 [7] – 1.16 [2]
16:0 Palmitic	18.4 [7] – 26.18 [2]
16:1 Palmitoleic	0.56 [2] – 1.00 [7]
18:0 Stearic	2.2 [7] – 2.88 [2]
18:1 Oleic	15.17 [2] – 19.94 [2]
18:2 Linoleic	49.07 [2] – 59.1 [7]
18:2 Gamma Linoleic	Not Available
18:3 Linolenic	0.23 [7]
20:0 Arachidic	0.41 [7]
22:0 Behenic	Not Available
Dihydrosterculic	Not Available
Malvalic	0.7 [8] – 1.5 [8]
Sterculic	0.3 [8] – 0.5 [8]
Minerals	
Calcium (% dwt)	0.1 [3] – 0.17 [6]
Copper (ppm dwt)	9.9 [3] – 54 [5]
Iron (ppm dwt)	67.0 [3] – 151 [5]
Magnesium (% dwt)	0.34 [3] – 0.37 [6]
Manganese (ppm dwt)	10 [5] – 20.1 [3]
Phosphorus (% dwt)	0.56 [9] – 0.75 [5]
Potassium (% dwt)	0.96 [3] – 1.21 [5]
Sodium (% dwt)	0.03 [3] – 0.31 [5]
Zinc (ppm dwt)	28.9 [3] – 37 [6]
Miscellaneous	
Gossypol, Free (% dwt)	0.59 [10] – 2.35 [10]
Gossypol, Total (% dwt)	0.80 [7] – 1.09 [7]
Vitamin (ppm)	
Vitamin E	99 [11] – 224 [11]

Appendix F: Appearance of Glyphosate Resistant Weeds

Appendix F: Appearance of Glyphosate Resistant Weeds

Monsanto considers product stewardship to be a fundamental component of customer service and business practices. The issue of glyphosate resistance is important to Monsanto because it can adversely impact the utility and life cycle of our products if it is not managed properly. The risk of weeds developing resistance and the potential impact of resistance on the usefulness of an herbicide vary greatly across different modes of action and are dependent on a combination of different factors. As leaders in the development and stewardship of glyphosate products for almost thirty years, Monsanto invests considerably in research to understand the proper uses and stewardship of the glyphosate molecule. This research includes an evaluation of some of the factors that can contribute to the development of weed resistance.

A. The Herbicide Glyphosate

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #: 1071-83-6), the active ingredient in the Roundup family of nonselective, foliar-applied, post-emergent agricultural herbicides, is among the world's most widely used herbicidal active ingredients. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate kills plant cells by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Franz et al., 1997). This aromatic amino acid pathway is not present in mammalian metabolic systems (Cole, 1985). This mode of action contributes to the selective toxicity of glyphosate toward plants and to the low risk to human health from the use of glyphosate according to label directions. A comprehensive human safety evaluation and risk assessment concluded that glyphosate has low toxicity to mammals, is not a carcinogen, does not adversely affect reproduction and development, and does not bioaccumulate in mammals (Williams et al., 2000). Glyphosate has favorable environmental characteristics, including a low potential to move to through soil to reach ground water and that it is degraded over time by soil microbes. Because it binds tightly to soil, glyphosate's bioavailability is reduced immediately after use, which is why glyphosate has no residual soil activity. An ecotoxicological risk assessment concluded that the use of glyphosate does not pose an unreasonable risk of adverse effects to non-target species, such as birds and fish, when used according to label directions (Giesy et al., 2000).

B. Characteristics Related to Resistance

Today, some 171 herbicide-resistant species and 286 biotypes within those species have been identified (Heap, 2004). Most of them are resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance usually has developed because of the long residual activity of these herbicides with the capacity to control weeds all year long and the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action. Using these criteria, and based on current use data, glyphosate is considered to be a herbicide with a low risk

for weed resistance (Benbrook, 1991). Nonetheless, a question has been raised as to whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide.

It is important to recognize that weed resistance is a herbicide-related issue, not a crop-related issue. The use of a specific herbicide with a herbicide tolerant crop is no different than the use of a selective herbicide over a conventional crop from a weed resistance standpoint. While the incidence of weed resistance is often associated with repeated applications of a herbicide product, its development depends very much on the specific herbicide chemistry in question as well as the plant's ability to inactivate them. Some herbicide products are much more prone to develop herbicide resistance than others. Glyphosate has been used extensively for three decades with very few cases of resistance development, particularly in relation to many other herbicides. A summary of some of those factors is described below.

B.1. Target Site Specificity

Target site alteration is a common resistance mechanism among many herbicide classes, such as acetolactate synthase-inhibitors and triazines, but is less likely for glyphosate.

An herbicide's mode of action is classified by the interference of a critical metabolic process in the plant by binding to a target protein and disrupting the required function. The "specificity" of this interaction is critical for the opportunity to develop target site mediated resistance. Because the herbicide contacts discreet amino acids during protein binding, changing one of these contact point amino acids can interrupt this binding. Specificity of inhibitor binding is dependent on the number and type of the amino acids serving as contact points and can be measured indirectly by counting the number of unique compounds that can bind in the same site. On one extreme, glyphosate is the only herbicide compound that can bind to EPSPS. Single amino acid substitutions near the active site have been observed for EPSPS, and while glyphosate binding is slightly weaker, these enzymes are also less fit. Similarly, high specificity is also observed for glutamine synthetase, binding three compounds including phosphinothricin in the active site (Crespo et al., 1999). Paraquat and diquat are the only two herbicides inhibiting photosystem I. No target site mutations have been reported to be responsible for resistance in these systems (Powles and Holtum, 1994).

On the other extreme are target enzymes that are efficiently inhibited by a wide array of compounds, e.g., acetolactate synthase (ALS) is inhibited by 53 and acetyl CoA carboxylase (ACCase) is inhibited by 21 separate herbicide compounds that bind both within and outside the active site (HRAC 2002; Trannel and Wright, 2002). These cases demonstrate that numerous non-critical amino acids are involved outside of the active site, offering a relatively large range of permissible mutations. In these two cases, a single amino acid change can result in virtual immunity to the class of herbicides and has directly led to the preponderance of resistant weed species for these mode-of-actions, 79 and 30 respectively.

Glyphosate competes for the binding site of the second substrate, phosphoenolpyruvate in the active site of EPSPS and is a transition state inhibitor of the reaction (Steinrucken and Amrhein, 1984). This was recently verified by x-ray crystal structure (Schonbrunn et al., 2001). As a transition state inhibitor, glyphosate binds only to the key catalytic residues in the active site. Catalytic residues are critical for function and cannot be changed without a lethal or serious fitness penalty. Furthermore, very few selective changes can occur near the active site of the enzyme to alter the competitiveness of glyphosate without interfering with normal catalytic function. Therefore, target site resistance is highly unlikely for glyphosate. This was further illustrated in that laboratory selection for glyphosate resistance using whole plant or cell/tissue culture techniques were unsuccessful (Jander et al., 2003; Widholm et al., 2001; OECD, 1999).

B.2. Limited Metabolism in Plants

Metabolism of the herbicide active moiety is often a principle mechanism for the development of herbicide resistance. The lack of glyphosate metabolism or significantly slow glyphosate metabolism has been reported in several species and reviewed in various publications (Duke, 1988; Coupland, 1985). Therefore, this mechanism is unlikely to confer resistance to glyphosate in plants.

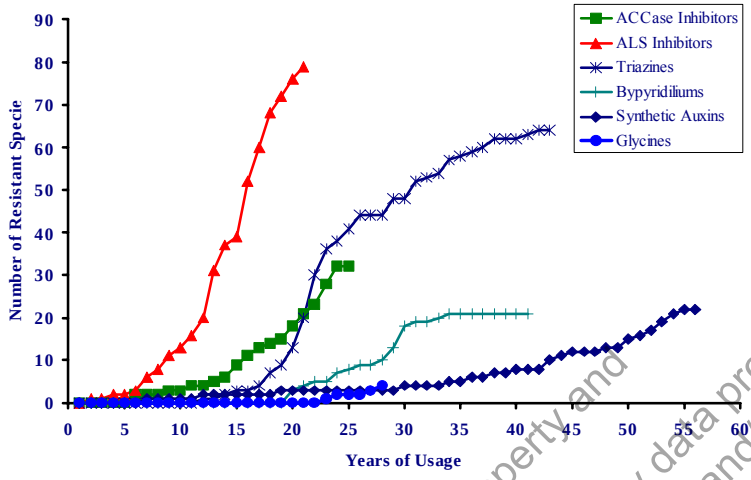
B.3. Lack of Soil Residual Activity

Herbicides with soil residual activity dissipate over time in the soil resulting in a sublethal exposure and in effect low dose selection pressure. Glyphosate adsorption to soils occurs rapidly, usually within one hour (Franz et al., 1997). Soil-bound glyphosate is unavailable to plant roots, so the impact of sublethal doses over time is eliminated. The postemerge-only activity of glyphosate allows for the use of a high dose weed management strategy.

The graph in Figure F-1 illustrates the instances of weed resistance to various herbicide families. The different slopes observed are largely due to the factors described above, which relate to chemistry and function, in addition to levels of exposure in the field. Glyphosate is a member of the glycine family of herbicides, which have experienced very limited cases of resistance despite almost three decades of use. The ALS inhibitors and triazine families, on the other hand, have experienced extensive cases of resistance even after they were available for only a relatively short period of time.

It is also important to recognize that each herbicide targets a large number of weeds, so the development of resistance in certain species does not mean the herbicide is no longer useful to the grower. For example, resistance of certain weeds to imidazolinone and sulfonyurea chemistries developed within three to five years of their introduction into cropping systems. Nevertheless, Pursuit (imidazolinone) herbicide had a 60% share of the U.S. soybean herbicide market despite the presence of a large number of resistant weeds because it was used in combination with other herbicides that controlled the resistant species. How weed resistance impacts the use of a particular herbicide varies greatly depending on the herbicide chemistry, the biology of the weed, availability of other control practices and the diligence with which it is managed.

Figure F-1. Number of Herbicide Resistant Weed Species Found By Years of Herbicide Family Use.



Heap, 2004

C. Weeds Resistant to Glyphosate

Weed resistance as generally defined as the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that should, under normal use conditions, effectively control that weed population. Thus, a resistant weed must demonstrate two criteria: 1) the ability to survive application rates of a herbicide product that once were effective in controlling it; and 2) the ability to pass the resistance trait to seeds. Procedures to confirm resistance generally require both field and greenhouse analyses, particularly if the level of resistance is relatively low. This correlation has been particularly important for the accurate detection of glyphosate resistance, for which the levels of resistance observed have been as low as 2X the susceptible biotypes.

Herbicide tolerance differs from resistance in that the species is not controlled but has the inherent ability to survive applications of the herbicide from the beginning. In other words, the species does not develop tolerance through selection but is innately tolerant.

As part of our product stewardship and customer service policy, Monsanto investigates cases of unsatisfactory weed control to determine the cause, as described in the performance evaluation program outlined in section E of this document. Weed control failures following application of Roundup agricultural herbicides are most often the result of management and/or environmental issues and are very rarely the result of herbicide resistance. The procedures included in Monsanto's performance evaluation program provide early detection of potential resistance, field and greenhouse protocols to

investigate suspected cases and mitigation procedures to respond to confirmed cases of glyphosate resistance.

To date, biotypes of only four weed species resistant to glyphosate have been identified and confirmed. In all cases, Monsanto worked with local scientists to identify alternative control options that have been effective in managing the resistant biotypes.

Lolium rigidum

In 1996 in Australia, it was reported that a biotype of annual ryegrass (*Lolium rigidum*) was surviving application of label recommended rates of glyphosate (Pratley et al., 1996). A collaboration was established with Charles Sturt University to develop an agronomic understanding of the biotype and investigate the mechanism of resistance. Where the biotype has been found, it has occurred within isolated patches within a field and does not appear to be widespread. The resistant biotype is easily controlled within conservation and conventional tillage systems with other herbicides, tillage or seed removal.

A large body of biochemical and molecular biology experiments between Australian ryegrass biotypes resistant and susceptible to glyphosate indicate that the observed resistance is due to a combination of factors. The mechanism of resistance appears to be multigenic and caused by a complex inheritance pattern, which is unlikely to occur across a wide range of other species. The mechanism is yet to be fully defined despite significant research effort; however, reduced cellular transport of glyphosate has been proposed (Lorraine-Colwill et al., 2003).

The resistant annual ryegrass biotype has also been observed in orchard systems of California and South Africa. Similar to the Australian locations, these fields are small and isolated. Monsanto established collaborations with local scientists to identify alternative control mechanisms, and the use of other herbicides, tillage, mowing, and seed removal have been very effective in controlling the ryegrass.

Annual ryegrass is not a common weed in cotton fields in the U.S.

Lolium multiflorum

A population of Italian ryegrass (*Lolium multiflorum*) was reported to survive labeled rates of glyphosate by a scientist conducting greenhouse and field trials in Chile. Monsanto conducted field and greenhouse trials to confirm the resistance and worked with the researcher to identify alternative control options. A population was also identified in Brazil. The resistant biotypes have been found on only a few farms and are easily controlled through tank mixes with other herbicides and cultural practices.

Italian ryegrass is not a common weed in cotton fields in the U.S.

Eleusine indica

A population of *Eleusine indica (*goosegrass*)* was reported to survive labeled rates of glyphosate in some orchard systems in Malaysia. Monsanto entered into collaborations with the University of Malaysia and identified alternative control options to effectively

manage the resistant biotype. Extensive molecular investigations determined that some of the resistant goosegrass plants have a modified EPSPS that is two to four times less sensitive to glyphosate than in more sensitive biotypes (Baerson et al., 2002). However, some resistant individuals did not exhibit the enzyme modification, suggesting that different mechanisms may be at play or resistance may be due to a combination of factors.

The resistant biotypes are easily controlled through application timing (applying glyphosate during the early growth stages), other herbicides, tillage and other cultural control practices.

Goosegrass is a warm season annual grass that can be found in U.S. cotton fields. Although considered a common weed species in cotton, it is not generally considered a troublesome weed (SWSS, 2001) and can be controlled effectively using a number of herbicide classes.

Conyza canadensis

Laboratory and field investigations confirmed the presence of a glyphosate-resistant biotype of marestail (*Conyza canadensis*) in certain states of the eastern and southern U.S. (VanGessel, 2001). The mechanism of resistance in the marestail biotype is currently under investigation. Findings thus far have been presented at regional and national weed science meetings and submitted for publication (Feng et al., 2004)

Investigations thus far indicate that this biotype has a heritable resistance ranging up to approximately six to eight times field herbicide application rates. Current data indicates that the heritance is dominant and transmitted by a singular nuclear gene. Resistance is not due to over-expression of EPSPS, glyphosate metabolism or reduction in glyphosate retention or uptake. Resistance is also not due to target site mutation, as the three isozymes of EPSPS identified in marestail were identical in sensitive and resistance lines. Our results demonstrate a strong correlation between impaired glyphosate translocation and resistance. Tissues from both sensitive and resistant biotypes showed elevated levels of shikimate, suggesting that EPSPS remained sensitive to glyphosate. Analysis of tissue shikimate levels relative to those of glyphosate demonstrated a reduced efficiency of EPSPS inhibition in the resistant biotypes. Our results are consistent with an exclusion mechanism for glyphosate resistance. Our current working hypothesis is that marestail resistance results from an alteration of glyphosate distribution that impairs its phloem loading and plastidic import.

The resistant marestail biotype has been observed in conventional and Roundup Ready cotton and soybean fields. As in other cases, Monsanto responded to weed control inquiries and alternative weed control options were provided. One of the most effective ways to minimize the resistant biotype is by planting a cover crop that can compete with marestail and limit its fall and winter germination. In addition, growers are advised to use a tank-mix of glyphosate with Clarity for cotton in their burndown treatment. If marestail is present in-crop, then growers are advised to use MSCA plus diuron in cotton.

In addition, as part of Monsanto's stewardship program, we have obtained a supplemental label, approved by EPA, which provides specific instructions on proper use of glyphosate herbicides in these counties where the resistant biotype has been confirmed. Growers in those counties are instructed to use the alternative control options, regardless of whether or not they had trouble controlling marestail on their farm the previous season, as a means to minimize spread of the resistant biotype. It has been recommended to growers in surrounding areas where the resistant biotype has not been confirmed that they use the alternative control options if marestail has been a difficult weed for them to control. This stewardship program has proven effective in controlling the glyphosate-resistant biotype and minimizing its spread beyond the southern and eastern regions of the U.S.

Other Species

Populations of two weed species in South Africa, hairy fleabane (*Conyza bonariensis*) and buckhorn plantain (*Plantago lanceolata*), have been reported to be resistant to glyphosate (Heap, 2004). Monsanto is investigating the populations and has not confirmed resistance at this time. Various herbicides are available for control of these species, but they do not commonly occur in U.S. cotton production.

Species that are tolerant to glyphosate, such as *Equisetum arvensis* (field horseweed), are occasionally described as "resistant." This characterization is technically inappropriate because glyphosate is not commercially effective on those weeds and they generally are not listed as controlled on Roundup agricultural herbicide product labels. Other species, such as *Convolvulus arvensis* (field bindweed) that are listed on the label may be partially tolerant or "difficult-to-control" with glyphosate alone. In these cases, additional herbicides are usually recommended to be tank-mixed with glyphosate. Still other species, such as *Abutilon theophrasti* (velvetleaf), may be listed as controlled by glyphosate on the label but a tank-mix recommendation for additional herbicide may be used in the field due to sensitive environmental or herbicide application conditions in certain counties or seasons.

In summary, Monsanto has effective product stewardship and customer service practices established to directly work with the grower communities and provide appropriate control measures for glyphosate-resistant weeds. Monsanto has collaborated with academic institutions to study these glyphosate-resistant biotypes and findings have been communicated to the scientific community through publications in peer-reviewed scientific journals and scientific meetings.

D. Weed management strategies for glyphosate

A key element of good weed management is using the correct rate of glyphosate at the appropriate window of application for the weed species and size present. Higher herbicide doses result in higher weed mortality and less diversity of resistance genes in the surviving population (Matthews, 1994). Low herbicide rates also may allow both heterozygous and homozygous resistant individuals to survive (Maxwell and Mortimer 1994), further contributing to the build up of resistant alleles in a population. As resistance is dependent upon the accumulation of relatively weak genes, which it appears,

may be the case for one or more of the four weed species that have evolved resistance to glyphosate, using a lethal dose of herbicide is critical.

Results that support these strategies are beginning to emerge from recent field research studies at several universities where it is documented that studies must be done in the field in the crop (Roush et al., 1990). Various weed management programs have been evaluated since 1998 to determine how they impact weed population dynamics. Studies were initiated in Colorado, Kansas, Nebraska, Wyoming ([redacted] 2002, unpublished data), and Wisconsin ([redacted] 2002) to evaluate continuous use of Roundup Ready technology with exclusive use of glyphosate or inclusion of herbicides with other modes-of-action, and rotation away from Roundup Ready technology. These treatment regimes were compared to a conventional herbicide program for each crop evaluated. General observations after five years are:

1. Use of a continuous Roundup Ready cropping system with either glyphosate alone at labeled rates or incorporation of herbicides with other modes-of-action resulted in excellent weed control with no weed shifts or resistance reported.
2. Use of glyphosate at below labeled rates resulted in a weed shift to common lambsquarters at two locations (NE, WY).
3. In WI, ALS resistant giant ragweed was selected for in the broad-spectrum residual herbicide regime implemented in the conventional corn cropping system. The continuous glyphosate system (using labeled rates) resulted in no significant weed shifts.

By using glyphosate at the recommended lethal dose, the build-up of weeds with greater inherent tolerance or any potential resistance alleles has been avoided over the duration of these studies. These results indicate that continuous Roundup Ready systems used over several years did not create weed shifts or resistant weeds when the correct rate of glyphosate was applied and good weed management was practiced.

E. Glyphosate stewardship program

Commercial experience, field trials and laboratory research demonstrate that one of the most important stewardship practices is achieving maximum control of the weeds. This can be accomplished by using the correct rate of glyphosate at the appropriate window of application for the weed species and size present, and using other tools or practices as necessary.

As the recognized leader in the development and commercialization of glyphosate, Monsanto is committed to the proper use and long-term effectiveness of glyphosate through a four-part stewardship program: developing appropriate weed control recommendations; continuing research to refine and update recommendations; educating growers on the importance of good weed management practices; and responding to repeated weed control inquiries through a performance evaluation program.

E.1. Develop Local Weed Management Recommendations to Ensure Maximum Practical Control is Achieved

Weed control recommendations in product labels and informational materials are based on local needs to promote the use of the management tool(s) that are most appropriate technically and economically for each region. Furthermore, growers are instructed to apply the same principles when making weed control decisions for the own farm operation. Multiple agronomic factors, including weed spectrum and population size, application rate and timing, herbicide resistance status (where applicable) and an assessment of past and current farming practices used in the region or on the specific operation are considered to ensure appropriate recommendations for the use of glyphosate to provide effective weed control. Carefully developing and regularly updating the use recommendations for glyphosate are fundamental to Monsanto's stewardship program.

Weed Spectrum

Weed spectrum refers to all of the weed species present in a grower's field and the surrounding areas that may impact those fields. The spectrum may vary across regions, farm operations, and even among fields within a farm operation depending on environmental conditions and other factors. Weed control programs should be tailored on a case by case basis by identifying the target weeds present, considering the efficacy of glyphosate and other weed management tools against those particular weeds, and assessing if any are unlikely to be controlled sufficiently with glyphosate alone (not included on the Roundup brand agricultural herbicide label; difficult to control based on the agronomic and/or environmental conditions; or documented resistance to glyphosate). A formulation, rate, application parameters and additional control tools are recommended as necessary to optimize control of all weeds in that system.

Application Rate

Application rate is integral to the correct use of glyphosate and critical to obtain effective weed control. Significant research is conducted to identify the appropriate rate of glyphosate that should be applied for a particular weed at various growth stages in various agronomic and environmental conditions. These rates are included in rate tables provided in product labels and other materials. In addition, Monsanto recommends that growers use the rate necessary to target the most difficult to control weed in his system to minimize weed escapes. When recommending tank mixes, growers should consider the potential impacts on glyphosate efficacy through antagonism or below-recommended rates and make adjustments accordingly.

ApplicationTiming

Application timing is based on the growth stage of weeds, the size/biomass of weeds and the agronomic and environmental conditions at the time of application. Delaying the application of glyphosate and allowing weeds to grow too large before applying the initial "recommended rate" of glyphosate will result in poor efficacy. Applying the glyphosate at a time while weeds are under agronomic stress (e.g., insect/disease) or environmental stress (e.g., moisture/cold) can also result in poor efficacy of control.

Compensating for a delayed application through subsequent applications may not be effective, as the first application may inhibit the growth of weeds and impair efficacy of the second application because the weeds may not be in an active growth process.

Correct application timing is dependent on the combined management of the weed spectrum, the size and layout of the farm operation and the feasibility to make timely applications of all weeds in the fields with labor and equipment available. Monsanto recommends an application timeline that targets susceptible growth stages of all weeds, and where applicable includes recommendations for inclusion of additional control tools as necessary to optimize control of all weeds on that farm.

Finally, it is important to assess the current agronomic practices used in that region or on that farm operation to integrate the glyphosate recommendations into the grower's preferred management system. Variables such as tillage methods, crop rotations, other herbicide programs, other agronomic practices, and the resistance status of the weeds to herbicides other than glyphosate can impact the spectrum of weeds present and the tools available to the grower.

Weed management recommendations communicated to the grower also incorporate other components of the glyphosate stewardship program including the use of certified seed, employing sanitary practices such as cleaning equipment between fields, and scouting fields and reporting instances of unsatisfactory weed control for follow up investigation.

E.2. Continuing Research

A fundamental component of Monsanto's leadership in glyphosate stewardship is continuing research on the recommended use of glyphosate and factors impacting its effectiveness. In addition to the extensive analyses conducted to determine the labeled rate of glyphosate prior to product registration, ongoing agronomic evaluations are conducted at the local level to refine weed management recommendations for specific weed species in specific locations.

Weed efficacy trials are part of ongoing efforts by Monsanto to tailor recommendations to fit local conditions and grower needs. Application rate and timing, additional control tools and other factors are included in these analyses. As a result of weed efficacy trials, changes are made to specific weed control recommendations where and when applicable, and modifications to local recommendations are highlighted to growers through informational sheets and other methods.

E.3. Education and Communication Efforts

Another key element of effective product stewardship and appropriate product use is education to ensure that growers understand and implement effective weed management plans and recommendations. Monsanto communicates weed management recommendations through multiple channels and materials to multiple audiences.

All internal technical and sales field representatives are required to take a weed management training course to understand the glyphosate stewardship program and the importance of proper product use. The training program is supported by ongoing weed management updates that highlight seasonal conditions and recommendations.

Monsanto weed management recommendations and the importance of sound agronomic practices are communicated to growers, dealers and retailers, academic extension and crop consultants through multiple tools:

a. Technology training programs; Highlighting weed management principles, weed management plans and practical management guidelines.
b. Technology use guide: Includes tables outlining appropriate rate and timing for different weed species and sizes.
c. Grower meetings: Conducted prior to planting to emphasize the importance of following local application recommendations.
d. Marketing programs: Designed to reinforce and encourage the continued adoption and use of weed management recommendations by the grower (e.g., recommended rate and timing of application, additional weed control tools when applicable).
e. Informational Sheets: Issued to growers and dealers/retailers to highlight local recommendations for specific weeds.

As with most stewardship efforts, education is key to help growers and other stakeholders understand the importance of proper product use and encourage those practices in the field.

E.4. Performance Inquiry Evaluation and Weed Resistance Management Plan

To support and enhance Monsanto's weed management principles and recommendations, Monsanto implements a performance evaluation program based on grower performance inquiries and field trial observations. The goal of the program is to continue to adapt, modify and improve Monsanto's weed control recommendations, with a focus on:

a. Particular weeds and growing conditions;
b. Providing product support to customers who are not satisfied with their level of weed control; and
c. Identifying and investigating potential cases of glyphosate resistance early so that mitigation strategies can be implemented.

The grower generally reports instances of unsatisfactory weed control following glyphosate application to Monsanto or the retailer. It is important to Monsanto, as part of its customer service and stewardship commitment, that these product performance inquiries are acted upon immediately, resolved to the satisfaction of the customer and not repeated.

The vast majority of inquiries is due to application error or environmental conditions and resolved through a phone conversation with the grower. However, a system is in place to investigate a repeated performance inquiry for a specific weed on a specific field within the same year. The investigation considers the various factors that could account for ineffective weed control such as:

a. Application rate and timing;
b. Weed size and growth stage;
c. Environmental and agronomic conditions at time of application;
d. Herbicide application calibration

In all cases, the first priority is to provide control options to the grower so that satisfactory weed control is achieved for that growing season. The majority of repeated product performance inquiries is due to improper application or environmental/agronomic conditions and not repeated. However, if the problem occurs again in that field and does not appear to be due to application or growing condition factors, then steps are taken to determine if resistance is the cause as outlined in the Monsanto Weed Resistance Management Plan.

The Monsanto Weed Resistance Management Strategy consists of three elements:

a. Identification process for potential cases of glyphosate resistance;
b. Initiation of steps to respond to cases of suspected resistance; and
c. Development and communication of guidelines to incorporate resistance mitigation into weed management recommendations.

Identification of potential cases of glyphosate resistance is accomplished through evaluation of product performance inquiries and local field trials. These efforts provide an early indication of ineffective weed control that may indicate potential resistance.

If the follow up investigation clearly indicates that the observation is due to application error or agronomic/environmental conditions, then appropriate control options are recommended to the grower for that season and the grower receives increased education on the importance of proper product use. The vast majority of weed control inquiries fall into this category.

If repeated lack of control is observed and does not appear to be due to application error or environmental conditions, then a field investigation is conducted by Monsanto to analyze control of the weed more thoroughly.

The vast majority of field investigations do not repeat the insufficient control reported by the grower, largely due to characteristics of the mode of action of glyphosate that make subsequent applications by the grower ineffective. The weed usually must be in an active growth phase in order for glyphosate to be effective, application error or environmental conditions that result in insufficient glyphosate to kill the weed often stunt its growth such that subsequent applications by the grower are ineffective. Monsanto's field investigations at this stage remove that artifact by ensuring that the weeds tested are in an

active growth phase. If the field investigation confirms that agronomic factors account for the observation, then the grower receives increased education on proper application recommendations.

In addition, the internal network of Monsanto technical managers and sales representatives in the surrounding area are notified to highlight any problematic environmental conditions or application practices that may be common in that area. Critical information regarding location, weed species, weed size, rate used and the potential reason for lack of control are captured, and the results are reviewed annually by the appropriate technical manager to identify any trends or learnings that need to be incorporated into the weed management recommendations.

If the reported observation is repeated in the field investigation, then a detailed performance inquiry is conducted and greenhouse trials are initiated. If greenhouse trials do not repeat the observation and the weed is clearly controlled at label rates, then a thorough follow up visit is conducted with the grower to review the application recommendations and conditions of his operation that may be impacting weed control. The internal network of agronomic managers is notified of the results to raise awareness of performance inquiries on that weed the following season. If the greenhouse efficacy trials do indicate insufficient control at label rates, then detailed studies are conducted to determine if the weed is resistant.

Resistance is considered to be confirmed if the two criteria outlined in the Weed Science Society of America definition of resistance are deemed to be fulfilled either through greenhouse data or experience with similar cases:

1) The suspect plant is demonstrated to tolerate labeled rates of glyphosate that previously were effective in controlling it; and
2) The suspect plant is capable of passing that ability to offspring (the trait is heritable).

Additional field trials will be initiated simultaneously as these investigations are conducted to identify the most effective and efficient alternative control options for that weed in various growing conditions. The research may be conducted internally as well as through collaboration with external researchers

If resistance is confirmed, then the scientific and grower communities are notified as appropriate and a weed resistance mitigation plan is implemented. The mitigation plan is designed to manage the resistant biotype through effective and economical weed management recommendations implemented by the grower. The scope and level of intensity of the mitigation plan vary depending on a combination of the following factors:

Biology and field characteristics of the weed (seed shed, seed dormancy, etc.);
Importance of the weed in the agricultural system;
Resistance status of the weed to other herbicides with alternate modes of action; and
Availability of alternative control options,

These factors are analyzed in combination with economic and practical management considerations to develop a tailored mitigation strategy that is technically appropriate for the particular weed and incorporates practical management strategies that can be implemented by the grower.

Once developed, the mitigation plan is communicated to the grower community through the use of supplemental labeling, informational fact sheets, retailer training programs, agriculture media or other means as appropriate.

The final step of the Weed Resistance Management Plan may include extensive genetic, biochemical or physiological analyses of confirmed cases of glyphosate resistance in order to elucidate the mechanism of resistance. Findings of this research are communicated to the scientific community through scientific meetings and publications, and information pertinent to field applications is incorporated into weed management recommendations.

F. Summary

Development of weed resistance is a complex process that is very difficult to accurately predict, and no single agronomic practice will mitigate resistance for all herbicides or all weeds. As a result, weed resistance needs to be managed on a case-by-case basis and tailored for the particular herbicide and grower needs. Using good weed management principles built upon achieving high levels of control through proper application rate, choice of cultural practices and appropriate companion weed control tools will allow glyphosate to continue to be used effectively.
The key principles for effective stewardship of glyphosate use, including Roundup Ready crops, include: 1) basing recommendations on local needs and using the tools necessary to optimize weed control; 2) proper rate and timing of application; and 3) responding rapidly to instances of unsatisfactory weed control.

Addendum to Monsanto's Request for a Determination of Nonregulated Status for Roundup Ready Flex Cotton MON 88913
Petition Number 04-086-01p
July 9, 2004

This addendum provides responses to APHIS/BRS' letter of clarification dated 22 June 2004, concerning Monsanto's request for a Determination of Nonregulated Status for Roundup Ready® Flex Cotton MON 88913.

APHIS/BRS Comment: Section V. Genetic Analysis

The border sequences of MON 88913 should be noted in the first paragraph on page 39.

Monsanto Response: The revised paragraph is presented below with additional text highlighted.

V. Genetic Analysis

Molecular analysis was performed to characterize the DNA insert in MON 88913. This analysis demonstrated that MON 88913 contains a single, intact insert comprised of two *cp4 epsps* gene expression cassettes of the T-DNA of plasmid PV-GHGT35, flanked by portions of the right and left border sequences derived from *Agrobacterium tumefaciens*: (1) the *ctp2/cp4 epsps* coding sequence whose transcription is directed by the FMV/TSF1 chimeric promoter, the leader (exon 1) and intron sequences from the *Arabidopsis thaliana tsf1* gene, and the transcriptional termination and polyadenylation sequence derived from the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1, 5-bisphosphate carboxylase (*rbc*) small subunit E9 gene; (2) a second *ctp2/cp4 epsps* coding sequence, identical to the first, whose transcription is directed by the 35S/ACT8 chimeric promoter, the leader, intron and flanking sequences from the *act8* gene of *Arabidopsis thaliana*, and the transcriptional termination and polyadenylation sequence derived from the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1, 5-bisphosphate carboxylase (*rbc*) small subunit E9 gene. This T-DNA was inserted into the cotton genome and results in the synthesis of a homogeneous CP4 EPSPS protein from the two *cp4 epsps* gene expression cassettes. The *ctp2* chloroplast transit peptide sequence, derived from the *Arabidopsis thaliana epsps* gene, is present to direct the CP4 EPSPS protein to the cotton chloroplast.

APHIS/BRS Comment: Section VII. Phenotypic Evaluation

APHIS provides guidance on agronomic performance data for upland cotton to be included in petitions for determination of non-regulated status. This document may be found on our website at http://www.aphis.usda.gov/brs/Cotton_ag.html. In the second paragraph on page 67 the petition groups agronomic characteristics into five categories. Two of the characteristics listed in our guidance document as suggested parameters (overwintering capacity of the plant and maturity) are not mentioned in the petition as one of the five categories. While they do not need to be presented as separate categories, they should be addressed in this sentence by adding them to one of the categories as appropriate.

While the aspects of maturity were adequately discussed in the petition, the issue of a change in the ability to overwinter was not presented. Please provide information that addresses this issue.

Monsanto Response 1: A paragraph from page 67 of the original petition has been modified to specifically reference the maturity and over-wintering characteristics and it is presented below. The added text is highlighted.

An evaluation of the phenotype of MON 88913 was conducted to assess the phenotypic equivalence to MON 88913(-). The phenotypic evaluation is based on laboratory and greenhouse experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of cotton. Comparisons of phenotypic parameters between MON 88913 and a negative segregant control, MON 88913(-), and also to conventional cotton were conducted to establish the phenotypic and seed compositional equivalence of MON 88913. In each of these assessments, MON 88913 was compared to the negative segregant control, MON 88913(-), which was derived from MON 88913 and thus possesses similar varietal background genetics to MON 88913. In evaluating the phenotypic characteristics of MON 88913, data were collected that address specific pest potential characteristics that are considered by USDA-APHIS. These phenotypic characteristics have been grouped into five general categories: 1) dormancy, germination and emergence; 2) vegetative growth; 3) reproductive growth, maturity and overwintering capacity; 4) seed retention on plant; and 5) plant interactions with disease, insect, and abiotic stressors.

Monsanto Response 2: Section VII.B.3 on pages 80 and 81 of the original petition has been modified to address the overwintering capacity of MON 88913. The revised section is presented below with the additional text highlighted:

VII.B.3. Confirmatory Field Observations and Overwintering Capacity

The agronomic evaluation of MON 88913 also included observational information on disease/pest susceptibility and phenotypic assessments from other product evaluation field trials conducted over several growing seasons (see Appendix A for a listing of USDA field trial final reports). These observations provide confirmatory information to the quantitative agronomic characterization data provided in this section (Section VII.). Field trials were conducted with MON 88913 during the years 2002-2003 under various product development protocols. These trials were established for the purpose of testing agronomic performance, crop efficacy and glyphosate tolerance, genetic background combining ability, developing weed control programs and assessing volunteer cotton incidence, assessing glyphosate residue levels, production of materials for product characterization studies, etc. The field designs and protocols for these trials varied according to purpose, with some trials replicated and others nonreplicated, most often comparing MON 88913 to MON 88913(-). Results of some of these trials have been presented at the Cotton Beltwide meetings (Subramani et al., 2002; May et al., 2003; Keeling et al., 2003; Martens et al., 2002; 2003; Croon et al., 2003).

In addition, in order to generate materials for *in planta* CP4 EPSPS protein characterization and quantification, molecular characterization, cottonseed composition, and evaluation of cottonseed dormancy, replicated field trials were conducted at four locations in the U.S. during 2002 (Appendix C, Section C.3.). The plants in these trials were grown under agronomic and cultural practices that are typical of cotton production within these regions. The field plots were periodically monitored (approximately every four weeks after planting) and observed for plant stressors, including susceptibility to common insect pests and pathogens. Insect pests were present at all sites and slight to moderate infestations were observed. Pesticides were applied in response to the insect pests according to normal agricultural practices for the location. Disease problems were not observed at any of the sites and there were no meaningful observed differences among the test, control and reference cotton plots with respect to arthropod damage.

Based upon the comparative evaluation of plant phenotypic characteristics between MON 88913 and MON 88913(-), and agronomic production practices[1], there is no reason to believe that the overwintering ability of MON 88913 is different from the control. In addition, there were no reports of overwintering plants from the agronomists who conducted the field trials in 2001 and 2002 and monitored the field plots after trial completion.

[1]U.S. production practice primarily involves cultivation of cotton as an annual species. Cotton plants are killed by freezing temperatures and/or cultural practices.

APHIS/BRS Comment: Section VII.C. Composition of Cottonseed of MON 88913

The second paragraph of this section on page 84 states that "MON 88913 was compared to MON 88913(-), which has background genetics representative of the test material but does not contain the DNA insert or produce the CP4 EPSPS protein." On page 85 as part of an explanation of the statistical differences of two fatty acid components in cottonseeds of MON 88913 and MON 88913(-), it reads that …"These differences could be explained by differences in the background genetics between MON 88913 and MON 88913(-)." Please explain this apparent contradiction.

Monsanto Response: The use of MON 88913(-) as an appropriate control for MON 88913 is explained in Section II.G. of the petition. In summary, MON 88913(-) is a negative segregant of MON 88913 and is expected to have background genetics which more closely approximate those of MON 88913 than the Coker 312 recipient variety. Cotton, unlike hybrid crops, is a varietal crop in the U.S., and exhibits a significant amount of seed-to-seed genetic variability within a given commercial variety. This variability is a natural genetic resource effectively utilized by commercial cotton breeders. Thus, the production of positive inbreds (test) and negative inbreds or true isolines (control), commonly utilized for hybrid crops, are not necessarily feasible for cotton. In this regard, taking advantage of conventional genetics, a negative segregant derived from MON 88913 was developed as an appropriate control [MON 88913(-)] for field tests and related product characterization studies. However, due the inherent natural variability within cottonseed, the genetic background of MON 88913(-) is expected to be very close, but not 100% identical, to that of MON 88913. Small differences in analyzed components between MON 88913 and MON 88913(-), such as those detected in the cottonseed composition analyses, can be explained by the inherent variability of the genetic background of cottonseed. More importantly, because the values for linoleic and oleic acid for MON 88913 and MON 88913(-) are within the natural variability (99% tolerance interval) measured for these components in conventional cottonseed, these differences are unlikely to be biologically meaningful.

APHIS/BRS Comment: Section VIII.D.6. Methods of Weed Control in Herbicide-Tolerant Cotton

The first paragraph of this section describes that glyphosate may be applied to Roundup Ready® cotton (event 1445) "…from emergence through the four-leaf stage. However, after the four-leaf stage up to layby (canopy closure into the row), the herbicide must be applied as a post-directed spray between the crop rows to minimize contact with the cotton plant and prevent potential crop injury." Is there any data on the quantity of glyphosate active ingredient used for post-directed application in comparison with over-the-top application?

Monsanto Response: We are not aware of any published data regarding the quantity of glyphosate active ingredient applied post-directed to the cotton plant compared to over-the-top applications. Monsanto has however surveyed growers regarding their usage of Roundup® agricultural herbicides on Roundup Ready cotton.

In 2002 an unpublished survey was conducted with over 500 U.S. cotton growers. Growers reported an average of 1.7 applications of a Roundup agricultural herbicide product following Roundup Ready cotton emergence, including both over-the-top and post-directed applications. In terms of the herbicide rate applied, growers reported applying an average of 0.76 and 0.74 lb ae/A glyphosate in first and second applications, respectively. The recommended labeled use rate for application at these times is 0.75 lb ae/A.

Finally, 97% of cotton growers surveyed made only one glyphosate application over-the-top (of Roundup Ready cotton) while only 39% of surveyed growers made a second application. According to label directions, a total of two over-the-top applications may be made to Roundup Ready cotton prior to the fifth true leaf. The fact that less than half of the growers made a second herbicide application indicates the difficulty they have in making a timely second application of Roundup herbicide over-the-top of Roundup Ready cotton, most often due to limiting environmental conditions (e.g. excessive wind or rainfall).

® Roundup is a registered trademark of Monsanto Technology LLC.

APHIS/BRS Comment: Has the use of Roundup Ready® cotton (event 1445), reduced the use of other herbicides? If so, please provide supportive data.

Monsanto Response: Roundup Ready cotton was introduced into the U.S. market in 1997. Since that time, a decrease in the use of a number of specific cotton herbicides has been observed. This can be determined by comparing USDA National Agricultural Statistics Service (NASS) upland cotton herbicide application data from the years 1997 and 2003. This data is summarized in the following table.

Of the cotton herbicide active ingredients that were being used in 1997, moderate decreases in use have been reported for metolachlor (-17.8%), trifluralin (-22.2%), pendimethalin (-25.6%), pyrithiobac-sodium (-25.9%) and prometryn (-28.1%). More significant reductions in use have been observed for fluometuron (-84.1%), MSMA/DSMA (-78.6%), cyanazine (-97.6%), norflurazon (-97.1%), clomazone (-96.7%) and the post-emergence graminicides (clethodim, fluazifop-p-butyl, quizalofop-ethyl, and sethoxydim) (-87.8%). Since its introduction into cotton in combination with BXN® cotton, bromoxynil use has also declined by 97.3% since its peak in 1999.

Glyphosate use in cotton has increased since the introduction of Roundup Ready cotton in 1997, with a corresponding increase in reduced- and no-till cotton practices in cotton. Other than glyphosate, diuron is the only other cotton herbicide with increased usage since 1997. This is likely the result of the voluntary withdrawal of the cyanazine (Bladex®) use label in cotton in the late 1990s.

® BXN is a registered trademark of Bayer Cropscience SA.

® Bladex is a registered trademark of E.I. du Pont de Nemours and Company.

Changes in Herbicide Use in Cotton Since 1997

Herbicide	2003		1997		% Change[b]
	Area Treated %	Total Applied[a]	Area Treated %	Total Applied[a]	
Glyphosate	70	13,637	14	1,599	752.9
Trifluralin	39	4,404	55	5,663	-22.2
Diuron	28	1,842	12	916	101.1
Pendimethalin	20	1,921	28	2,583	-25.6
Pyrithiobac-sodium	12	131.4	23	177.3	-25.9
Prometryn	11	1,245	19	1,731	-28.1
Fluometuron	8	800	44	5,026	-84.1
MSMA/DSMA	7	1,245	33	5,817	-78.6
Metolachlor	5	626[c]	5	762	-17.8
Cyanazine	<0.5	55.1	18	2,283	-97.6
Norflurazon	<0.5	30.7	13	1,077	-97.1
Clomazone	<0.5	17.0	8	518	-96.7
Bromoxynil	<0.5	16.0	7[d]	593[d]	-97.3
Graminicides[e]	<0.5	14.8	7	121	-87.8
States surveyed[1,2]	AL, AZ, AR, CA, GA, LA, MS, MO, NC, SC, TN, TX		AL, AZ, AR, CA, GA, LA, MS, MO, NC, SC, TN, TX		
Acreage represented[1,2]	12,795,000		13,075,000		
Total planted cotton acreage[3,4]	13,301,000		13,558,000		

[a] 1000 lbs. Calculated values adjusted to reflect total upland cotton acreage planted for respective years.

[b] Percent change to total applied (lbs.). Calculated values adjusted to reflect total upland cotton acreage planted. Values normalized to 1997 upland cotton planted acreage.

[c] Includes both racemic and S-forms of metolachlor.

[d] Bromoxynil calculated values based upon 1999 upland cotton planted acreage[5,6].

[e] Clethodim, fluazifop-p-butyl, quizalofop-ethyl, and sethoxydim

1. USDA-NASS. 2004. Agricultural Chemical Usage 2003 Field Crops Summary. Pp 93-94. Agricultural Statistics Board.

2. USDA-NASS. 1998. Agricultural Chemical Usage 1997 Field Crops Summary. Pp 24-25. Economics Research Service.

3. USDA-NASS. 2004. Crop Production – Acreage. P 18. Agricultural Statistics Board.

4. USDA-NASS. 1998. Crop Production – Acreage. P 23. Agricultural Statistics Board.

5. USDA-NASS. 2000. Agricultural Chemical Usage 1999 Field Crops Summary. Pp 33-34. Agricultural Statistics Board.

6. USDA-NASS. 2000. Crop Production – Acreage. P 19. Agricultural Statistics Board.

APHIS/BRS Comment: Appendix A: USDA Notifications, Table A-1. MON 88913 Field Trial Notification Numbers.

The table lists notifications of the MON 88913 event. However, in reviewing the field data reports in the notification files, the trials listed are for other event numbers. During a telephone conversation with you on May 17, 2004, you suggested that MON 88913 may have been renumbered from 9910. Upon further review that appears to be the case, but four of the notifications are not for either 9910 or 88913. These notifications are 00-089-13n, 00-118-10n, 00-140-06n, and 00-213-01n. It should also be noted that there were four notifications that were never planted. These notifications are 00-022-50n, 00-038-23n, 00-042-02n, and 00-059-06n. APHIS has not received field data reports for about half of the notifications listed in the table, but this is understandable because many of the reports are not yet due. However, the report for notification 01-232-01r is long overdue.

For this table, please provide the following information.

1. Clarify and verify the number change from 9910 to MON 88913, and that they are in fact the same event.

2. Determine if the four notifications listed above are for events other than 9910 or MON 88913. If so, provide assurance that data collected from these events were not used to support the petition for nonregulated status.

3. If possible, please provide the field data report for notification 01-232-01n.

Monsanto Response to 1: MON 88913 and 9910 refer to the same transformation event. Within Monsanto, new agricultural biotechnology candidates are assigned an identification number during the research stage. For this product, the research number was 9910. A MON number (e.g. MON 88913) is assigned later in the development stage. Because field trials of MON 88913 were conducted over a period of several years, a portion of the notifications listed in Table A-1 were granted for "9910" and others were granted for "MON 88913".

Monsanto Response to 2: The first five notifications listed under 2000 field trials in Table A-1 were granted for regulated glyphosate-tolerant cotton plants produced from the same transformation plasmid used in developing MON 88913, but these field trials did not specifically include MON 88913. The inclusion of these notifications in the table was due to an oversight. The data from these trials were not used to support Petition No. 04-086-01p.

Therefore, the following notifications should be removed from Table A-1:
00-038-23n
00-042-02n
00-059-06n
00-089-13n
00-118-10n

Both 00-140-06n and 00-213-01n list the event 9910 in the notification. Monsanto acknowledges that several of the notifications listed in Table A-1 were not planted. Notification 00-022-50n is not a Monsanto notification.

Monsanto Response to 3: Notifications 01-232-01r and 01-232-01n are not Monsanto notifications.

APHIS/BRS Comment: There were also a few typographical errors that the reviewers noticed. Please check them and acknowledge if you agree with them.

Monsanto Response: Monsanto agrees with all of the suggested typographical corrections.